PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/54, 15/82, 9/10, C08B 30/00, A01H 5/00, A23L 1/0522

A2

(11) International Publication Number: WO 97/22703

(43) International Publication Date: 26 June 1997 (26.06.97)

(21) International Application Number: PCT/US96/19678

(22) International Filing Date: 12 December 1996 (12.12.96)

(30) Priority Data:
60/009,113 20 December 1995 (20.12.95) US

(71) Applicant *(for all designated States except US)*: E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: HUBBARD, Natalie, Louise [US/US]; 2204 Mill Lane, Wilmington, DE 19810-4028 (US). KLEIN, Theodore, Mitchell [US/US]; 2229 Rosewood Drive, Wilmington, DE 19810-2815 (US). BROGLIE, Karen, E. [US/US]; 520 Port Royal Court, Landenberg, PA 19350-1333 (US).

(74) Agent: MORRISSEY, Bruce, W.; E.I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).

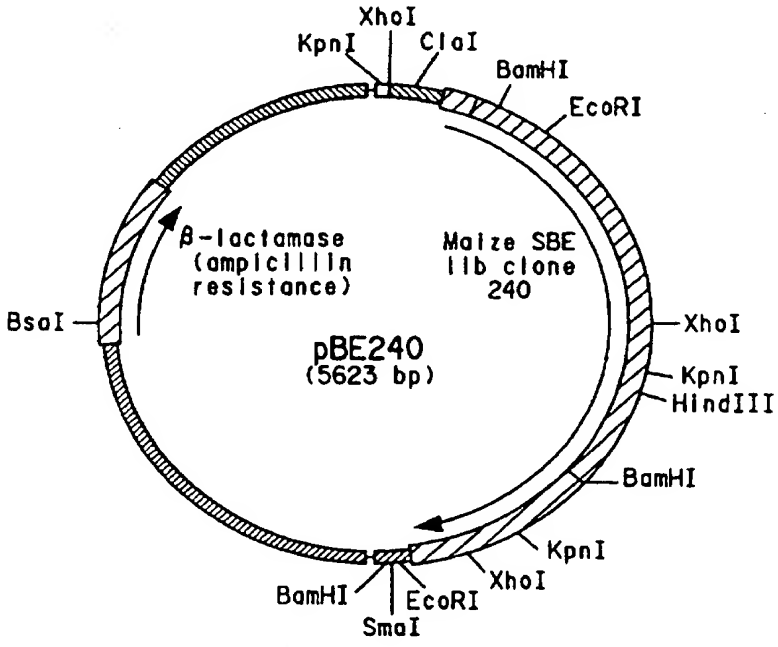
(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, HU, IL, IS, JP, KG, KP, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, TR, TT, UA, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.
With an indication in relation to a deposited microorganism furnished under Rule 13bis separately from the description.
Date of receipt by the International Bureau:
25 February 1997 (25.02.97)

(54) Title: NOVEL STARCHES VIA MODIFICATION OF EXPRESSION OF STARCH BIOSYNTHETIC ENZYME GENES

(57) Abstract

The instant invention discloses utilization of a cDNA clone to construct sense and antisense genes for inhibition of starch branching enzyme enzymatic activity in corn. More specifically, this invention concerns a method of controlling the starch fine structure of starch derived from the grain of corn comprising: (1) preparing a chimeric gene comprising a nucleic acid fragment encoding a starch branching enzyme structural gene or a fragment thereof, operably linked in either sense or antisense orientation on the upstream side to a nucleic acid fragment encoding a promoter that directs gene expression in corn endosperm tissue, and operably linked on the downstream side to a nucleic acid fragment encoding a suitable regulatory sequence for transcriptional termination, and (2) transforming corn with said chimeric gene, wherein expression of said chimeric gene results in alteration of the fine structure of starch derived from the grain of said transformed corn compared to the fine structure of starch derived from corn not possessing said chimeric gene.



TITLE

NOVEL STARCHES VIA MODIFICATION OF EXPRESSION OF STARCH BIOSYNTHETIC ENZYME GENES

BACKGROUND OF THE INVENTION

Characteristics and Commercial Utility of Starch

Starch is a mixture of two polysaccharides, amylose and amylopectin. Amylose is an unbranched chain of up to several thousand α–D-glucopyranose units linked by α-1,4 glycosidic bonds. Amylopectin is a highly branched molecule made up of up to 50,000 α-D-glucopyranose residues linked by α-1,4 and α-1,6 glycosidic bonds. Approximately 5% of the glycosidic linkages in amylopectin are α-1,6 bonds, which leads to the branched structure of the polymer.

Amylose and amylopectin molecules are organized into granules that are stored in plastids. The starch granules produced by most plants are 15-30% amylose and 70-85% amylopectin. The ratio of amylose to amylopectin and the degree of branching of amylopectin affects the physical and functional properties of the starch. Functional properties, such as viscosity and stability of a gelatinized starch determine the usefulness and hence the value of starches in food and industrial applications. Where a specific functional property is needed, starches obtained from various crops such as corn, rice, or potatoes may meet the functionality requirements. If a starch does not meet a required functional property, if for example it must have stable viscosity under high temperatures and acidic conditions, the functionality can sometimes be achieved by chemically modifying the starch. Various types and degrees of chemical modification are used in the starch industry, and the labeling and use of chemically modified starches must meet government regulations.

Within the starch bearing organs of plants, the proportion of amylose to amylopectin and the degree of branching of amylopectin are under genetic control. For example, plants homozygous recessive for the *waxy* (*wx*) gene lack a granule-bound starch synthase enzyme and produce nearly 100% amylopectin. Plants homozygous recessive for the *amylose extender* (*ae*) gene can produce starch granules that are up to 90% amylose (see U. S. Pat. No. 5,300,145). The *dull* gene has been shown to influence the levels of activity of a starch synthase and a starch branching enzyme.

Most cereal crops are handled as commodities, and many of the industrial and animal feed requirements for these crops can be met by common varieties which are widely grown and produced in volume. However, there exists at present a growing market for crops with special end-use properties which are not met by grain of standard composition. Most commonly, specialty corn is differentiated from "normal" corn, also known as field corn, by altered endosperm properties, such as an overall change in the ratio of amylose to amylopectin as in waxy or high amylose corn, an increased

The use of chemically modified starches in the United States is regulated by the Food and Drug Administration (FDA). "Food starch-modified" starches may be used in food but must meet specified treatment limits, and "industrial starch-modified" starches may be used in items such as containers that come in contact with food and must also meet specified treatment requirements; Code of Federal Regulations, Title 21, Chapter 1, Part 172, Food Additives Permitted in Food for Human Consumption, Section 172, 892, Food Starch-Modified, U. S. Government Printing Office, Washington, D. C. 1981; (a) Part 178, Indirect Food Additives, Sect. 178.3520, Industrial Starch-Modified. These regulations limit the degree of chemical modification by defining the maximum amount of chemical reagent that can be used in the modification steps. The levels of by-products in starch resulting from the modification process are also regulated. For example, propylene chlorohydrin residues in hydroxypropyl starch are of special concern; Tuschhoff, J. V., (1986) Hydroxypropylated Starches, In Modified Starches: Properties and Uses, O. B. Wurzburg, ed., CRC Press, Boca Raton, FL, pp. 55-57.

Alteration of Starch Fine Structure Through Molecular Genetic Manipulation of Starch-Bearing Plants

Differences in the degree of starch branching or polymerization are known to result in a change in the physiochemical properties of starch. It has been suggested that starches, tailor-made for specific applications, may be generated by alteration of the branch chain distribution of the amylopectin molecule, the relative proportion of amylose to amylopectin or the degree of polymerization of amylose. However, achieving phenotypic alteration of starch composition has been problematic; while key enzymes in starch biosynthesis have been identified, their exact roles remain uncertain. Thus, correlation of activities of particular enzymes with particular molecular characteristics of starch structure and, in turn, with starch function in food and industrial products has been difficult. Although desirable functional properties that an ideal starch might need can be envisioned, there is only a vague understanding of what the molecular structure of the starch should be to achieve this and little understanding of how particular starch biosynthetic enzymes specifically affect those parameters. For example, the role of individual enzymes in determining the branching patterns and length of branches is as yet unclear and is compounded by the lack of understanding of how branching enzymes and starch synthases interact.

WO 94/09144 discusses the generation of plants with improved ability to synthesize starch at elevated temperatures. This publication proposes that the limiting factor in grain filling at elevated temperature is the lability of certain starch biosynthetic enzymes, particularly starch synthase (SS) and starch branching enzyme (SBE). The

Alteration of starch fine structure in corn is complicated by the fact that three SBE isoforms have been identified. In corn endosperm, the three isoforms that demonstrate starch branching enzyme activity are SBEI, SBEIIa and SBEIIb. In the *amylose extender* (*ae*) mutant, SBEIIb activity has been found to be deficient while in the *dull* (*du*) mutant, decreased levels of SBEIIa are observed (Boyer, C. D. and Preiss, J. (1981) *Plant Physiol.* 67:1141-1145). Studies of the catalytic properties of the corn starch branching enzymes indicate that the isoforms differ in substrate preference and in the length of glucan chain that is transferred. SBEI activity is higher when amylose serves as the substrate, and longer chains are preferentially transferred. The SBEII isoforms display higher activity with more highly branched substrates such as amylopectin. These enzymes preferentially transfer shorter glucan chains (Guan et al. (1993) *Plant Physiol.* 102:1269-1273; Takeda et al. (1993) *Carbohydrate Res.* 240:253-263).

A corn SBEI cDNA has been cloned and sequenced (Baba et al. (1991) *Biochem. Biophys. Res. Commun.* 181:87-94; Fisher et al. (1995) *Plant Physiol.* 108:1313-1314). In addition, a corn SBEII cDNA clone has been isolated and the nucleotide sequence of the clone has been published (Fisher et al. (1993) *Plant Physiol.* 102:1045-1046). This cDNA clone maps to the *ae* locus, confirming that this locus encodes the structural gene for corn SBEIIb (Stinard et al. (1993) *Plant Cell* 5:1553-1566).

Starch isolated from the *ae* mutant is known to differ in structure from that isolated from dent corn (Baba et al. (1984) *Agric. Biol. Chem.* 48:1763-1775). The effect of the *ae* allele on starch properties has been investigated (Yamada et al. (1978) *Starke* 30:145-148). Increasing doses of *ae* in a *waxy* (*wx*) background produce an increase in the gelatinization temperature so that for the homozygous mutant, incomplete cooking of the starch is observed, even at 95°C. These authors indicate that the increase in viscosity associated with *ae wx* starch is highly desirable and suggest a "target" starch with properties intermediate between *wx* and *ae wx*. While mutations which influence the levels of corn SBEIIa and SBEIIb are available, mutations in the SBEI structural gene have yet to be identified. The lack of SBEI mutants may indicate that the absence of this branching enzyme isoform is lethal to the plant. Alternatively, a SBEI null mutation may give rise to no observable change in seed phenotype or one that is not readily distinguished from existing starch mutants.

Molecular genetic solutions to the generation of starches from corn with altered fine structures have a decided advantage over more traditional plant breeding approaches. Changes to starch fine structure can be produced by specifically inhibiting expression of one or more of the SBE isoforms by antisense inhibition or cosuppression.

function in glucan chain elongation, it is impossible to make predictions concerning starch structure based upon the catalytic properties of each isoform.

SUMMARY OF THE INVENTION

The instant invention discloses utilization of a cDNA clone to construct sense and antisense genes for inhibition of starch branching enzyme enzymatic activity in corn grain or endosperm. More specifically, this invention concerns a method of controlling the branch chain distribution of the amylopectin, the relative proportion of amylose to amylopectin and the degree of polymerization of amylose components of starch in corn comprising: (1) preparing a chimeric gene comprising a nucleic acid fragment encoding a starch branching enzyme structural gene or a fragment thereof, operably linked in either sense or antisense orientation on the upstream side to a nucleic acid fragment that encodes a promoter that directs gene expression in corn endosperm tissue, and operably linked on the downstream side to a nucleic acid fragment encoding a suitable regulatory sequence for transcriptional termination, and (2) transforming corn with said chimeric gene, wherein expression of said chimeric gene results in alteration of the branch chain distribution of the amylopectin molecular component of starch derived from the grain of said transformed corn compared to the branch chain distribution of the amylopectin molecular component of starch derived from corn not possessing said chimeric gene. This invention also concerns corn varieties prepared by transformation using said method, starch isolated from the grain of a corn variety prepared using said method, and a method of preparing a thickened foodstuff comprising combining a foodstuff, water, and an effective amount of a starch isolated from the grain of a corn variety prepared using the said method, and cooking the resulting composition as necessary to produce said thickened foodstuff.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE DESCRIPTIONS

The invention can be more fully understood from the following detailed description and the accompanying drawings and the sequence descriptions which form a part of this application.

Figure 1 presents a restriction map of plasmid pBE240 that contains a cDNA insert comprising 78 bp of 5' untranslated DNA, a 2397 bp open reading frame encoding the corn SBEIIb coding region and 190 bp of 3' untranslated DNA.

Figure 2 is a restriction map of plasmid pBE44 comprising a 414 bp 3' fragment of the insert of pBE240 in antisense orientation with respect to the corn 27 kd zein promoter.

Figure 3 is a restriction map of plasmid pML103, used as an intermediate cloning vehicle in construction of chimeric genes of the instant invention.

Figure 15 is a restriction map of plasmid pBE111 comprising a 1810 bp cDNA fragment encoding a truncated SBEI joined in sense orientation with respect to the maize 27 kd zein promoter.

Figure 16 compares RVA profiles of starch from waxy kernels, kernels homozygous for amylose extender (*ae*) and waxy and from kernels containing the pBE44 construct plus waxy. Viscosity, in stirring number units (SNU), and temperature (degrees Celsius) have been measured and plotted as a function of time (in minutes).

SEQ ID NO:1 depicts the nucleotide sequence of the cDNA insert in plasmid pBE240 and the corresponding amino acid sequence of the entire corn SBEIIb enzyme.

SEQ ID NO:2 depicts the nucleotide sequence of the the 414 bp insert of pBE44.

SEQ ID NOS:3 and 4 depict the PCR primers BE41 and BE42 used for preparation of the 414 bp insert of pBE44.

SEQ ID NO:5 depicts the nucleotide sequence of the the 507 bp insert of pBE43.

SEQ ID NOS:6 and 7 depict the PCR primers BE39 and BE40 used for preparation of the 507 bp insert of pBE43.

SEQ ID NO:8 depicts the nucleotide sequence of the the 2165 bp insert of pBE45.

SEQ ID NO:9 depicts the nucleotide sequence of the the 2087 bp insert of pBE96.

SEQ ID NOS:10 and 11 depict the PCR primers BE14 and BE15 used for preparation of the probe used to isolate the 2772 bp insert of pBE65. BE15 (SEQ ID NO:11) was also used for the preparation of the insert in plasmid pBE79.

SEQ ID NO:12 depicts the nucleotide sequence of the the 2772 bp insert of pBE65.

SEQ ID NO:13 depicts the nucleotide sequence of the the 373 bp insert of pBE68.

SEQ ID NOS:14 and 15 depict the PCR primers BE43 and BE52 used for preparation of the 373 bp insert of pBE68.

SEQ ID NO:16 depicts the nucleotide sequence of the the 571 bp insert of pBE69.

SEQ ID NOS:17 and 18 depict the PCR primers BE46 and BE50 used for preparation of the 571 bp insert of pBE69.

SEQ ID NO:19 depicts the nucleotide sequence of the the 2487 bp insert of pBE72.

SEQ ID NO:20 depicts the nucleotide sequence of the the 1865 bp insert of pBE97.

the repeating crystalline and amorphous units in the starch granule is quite regular with a repeat distance of 9 nm observed in starch from a wide variety of plant species (Jenkins (1993) Starch/Starke 45:417-420). Thus A and B1 chains are less than 9nm in length B2 and B3 chains are between 18 and 27 nm in length and B4+ chains are greater than 36 nm.

As used herein, the term "nucleic acid" refers to a large molecule which can be single-stranded or double-stranded, composed of monomers (nucleotides) containing a sugar, phosphate and either a purine or pyrimidine. A "nucleic acid fragment" is a fraction of a given nucleic acid molecule. In higher plants, deoxyribonucleic acid (DNA) is the genetic material while ribonucleic acid (RNA) is involved in the transfer of the information in DNA into proteins. A "genome" is the entire body of genetic material contained in each cell of an organism. The term "nucleotide sequence" refers to a polymer of DNA or RNA which can be single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases capable of incorporation into DNA or RNA polymers.

As used herein, "essentially similar" refers to DNA sequences that may involve base changes that do not cause a change in the encoded amino acid, or which involve base changes which may alter one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence. It is therefore understood that the invention encompasses more than the specific exemplary sequences. Modifications to the sequence, such as deletions, insertions, or substitutions in the sequence which produce silent changes that do not substantially affect the functional properties of the resulting protein molecule are also contemplated. For example, alteration in the gene sequence which reflect the degeneracy of the genetic code, or which results in the production of a chemically equivalent amino acid at a given site, are contemplated; thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another hydrophobic amino acid residue such as glycine, valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a biologically equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein. In some cases, it may in fact be desirable to make mutants of the sequence in order to study the effect of alteration on the biological activity of the protein. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products.

ribozyme sequences that may increase the efficacy of antisense RNA to block gene expression. "Ribozyme" refers to a catalytic RNA and includes sequence-specific endoribonucleases.

As used herein, suitable "regulatory sequences" refer to nucleotide sequences located upstream (5'), within, and/or downstream (3') to a coding sequence, which control the transcription and/or expression of the coding sequences. These regulatory sequences include promoters, translation leader sequences, transcription termination sequences, and polyadenylation sequences. In artificial DNA constructs, regulatory sequences can also control the transcription and stability of antisense RNA.

"Promoter" refers to a DNA sequence in a gene, usually upstream (5') to its coding sequence, which controls the expression of the coding sequence by providing the recognition for RNA polymerase and other factors required for proper transcription. A promoter may also contain DNA sequences that are involved in the binding of protein factors which control the effectiveness of transcription initiation in response to physiological or developmental conditions. It may also contain enhancer elements.

An "enhancer" is a DNA sequence which can stimulate promoter activity. It may be an innate element of the promoter or a heterologous element inserted to enhance the level and/or tissue-specificity of a promoter. "Constitutive" promoters refer to those that direct gene expression in substantially all tissues and demonstrate little temporal or developmental regulation. "Organ-specific" or "development-specific" promoters as referred to herein are those that direct gene expression almost exclusively in specific organs, such as leaves or seeds, or at specific developmental stages in an organ, such as in early or late embryogenesis, respectively.

The term "operably linked" refers to nucleic acid sequences on a single nucleic acid molecule which are associated so that the function of one is affected by the other. For example, a promoter is operably linked with a structural gene (i.e., a gene encoding a starch branching enzyme) when it is capable of affecting the expression of that structural gene (i.e., that the structural gene is under the transcriptional control of the promoter).

The term "expression", as used herein, is intended to mean the production of a functional end-product encoded by a gene. More particularly, "expression" refers to the transcription of the sense (mRNA) or the antisense RNA derived from the nucleic acid fragment(s) of the invention that, in conjuction with the protein apparatus of the cell, results in altered levels of protein product. "Antisense inhibition" refers to the production of antisense RNA transcripts capable of preventing the expression of the target protein. "Overexpression" refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal or non-transformed

A number of genes encoding carbohydrate branching enzymes have been isolated and sequenced. These include glycogen branching enzymes from *Saccharomyces cerevisiae* (Thon et al. (1992) *J. Biol. Chem.* 267:15224-15228), *E. coli* (Baecker et al. (1986) *J. Biol. Chem.* 261:8738-8743), *Bacillus stearothermophilus* (Kiel et al. (1991) *Mol. Gen. Genet.* 230:136-144), *Bacillus caldolyticus* (Kiel et al. (1992) *DNA Seq.* 3: 221-232), human (Thon et al. (1993) *J. Biol. Chem.* 268:7509-7513), *Aspergillus nidulans* (Kiel et al. (1990) *Gene* 89:77-84), *Streptomyces coelicolor* (EMBL accession number X73903), *Streptomyces aurofaciens* (Homerova, D. and Kormanec, J. (1994) *Biochem. Biophys. Acta* 1200:334-336) and starch branching enzymes from corn (Baba et al., (1991) *Biochem. Biophys. Res. Commun.* 181:87-94; Fisher et al. (1993) *Plant Physiol.* 102:1045-1046; Fisher et al. (1995) *Plant Physiol.* 108:1313-1314), pea (Burton et al. (1995) *Plant J.* 7:3-15), potato (Poulsen, P. and Kreiberg, J. D. (1993) *Plant Physiol.* 102:1053-1054), cassava (Salehuzzaman et al. (1992) *Plant Mol. Biol.* 20:809-819), rice (Kawasaki et al. (1993) *Mol. Gen. Genet.* 237:10-16; Mizuno et al. (93) *J. Biol. Chem.* 268:19084-19091) and *Arabidopsis thaliana* (EMBL accession numbers U18817 and U22428). Preferred among these are the corn starch branching enzyme genes. These genes can be isolated by techniques routinely employed by the skilled artisan for isolation of genes when the nucleotide sequence of the desired gene is known, or when the sequence of a homologous gene from another organism is known. Sequence information about the desired gene can be used to prepare oligonucleotide probes for identification and isolation of the entire branching enzyme gene from an appropriate genetic library. This library may be a genomic library, wherein the coding region may be contained on a single DNA fragment or may be contained on several distinct DNA fragments. Moreover, two or more exons encoding the branching enzyme may be separated by one or more introns. Alternatively, the library may be a cDNA library wherein the liklihood of isolating a cDNA clone comprising the entire coding region as one contiguous sequence is greater. In either instance, the appropriate clone(s) can be identified by DNA-DNA hybridization with probes corresponding to one or more portions of the desired genes. Alternatively, oligonucleotide primers can be prepared and employed as PCR primers in order to amplify and subsequently isolate all or part of the branching enzyme coding region from genomic DNA, or from the genomic or cDNA libraries described above.

Several different assays can be used to measure branching enzyme activity. In the phosphorylase stimulation assay (Boyer, C. D. and Preiss, J. (1978) *Carbohydr. Res.* 61:321-334), activity is measured indirectly by following the ability of branching enzymes to stimulate formation of α–D-glucan from glucose-1-phosphate by

Promoters utilized to drive gene expression in transgenic plants can be derived from many sources so long as the chosen promoter(s) have sufficient transcriptional activity to accomplish the invention by expressing translatable mRNA or antisense RNA in the desired host tissue. For example, promoters for expression in a wide array of plant organs include those directing the 19S and 35S transcripts in Cauliflower mosaic virus (Odell et al. (1985) *Nature* 313:810-812; Hull et al. (1987) *Virology* 86:482-493), small subunit of ribulose 1,5-bisphosphate carboxylase (Morelli et al. (1985) *Nature* 315:200-204; Broglie et al. (1984) *Science* 224:838-843; Hererra-Estrella et al. (1984) *Nature* 310:115-120; Coruzzi et al. (1984) *EMBO J.* 3:1671-1679; Faciotti et al. (1985) *Bio/Technology* 3:241 and chlorophyll a/b binding protein (Lamppa et al. (1986) *Nature* 316:750-752).

Depending upon the application, it may be desirable to select promoters that are specific for expression in one or more organs of the plant. Examples include the light-inducible promoters of the small subunit of ribulose 1,5-bisphosphate carboxylase, if the expression is desired in photosynthetic organs, or promoters active specifically in seeds.

Preferred promoters are those that allow expression specifically in seeds. This may be especially useful, since seeds are the primary location of long-term starch accumulation. In addition, seed-specific expression may avoid any potential deleterious effects that branching enzyme modulation may have on non-seed organs. Examples of seed-specific promoters include, but are not limited to, the promoters of seed storage proteins. The seed storage proteins are strictly regulated, being expressed almost exclusively in seeds in a highly organ-specific and stage-specific manner (Higgins et al. (1984) *Ann. Rev. Plant Physiol.* 35:191-221; Goldberg et al. (1989) *Cell* 56:149-160; Thompson et al. (1989) *BioEssays* 10:108-113). Moreover, different seed storage proteins may be expressed at different stages of seed development.

There are currently numerous examples for seed-specific expression of seed storage protein genes in transgenic plants. These include genes from monocotyledonous plants such as for barley β-hordein (Marris et al. (1988) *Plant Mol. Biol.* 10:359-366) and wheat glutenin (Colot et al. (1987) *EMBO J.* 6:3559-3564). Moreover, promoters of seed-specific genes, operably linked to heterologous coding sequences in chimeric gene constructs, also maintain their temporal and spatial expression pattern in transgenic plants. Such examples include linking either the Phaseolin or Arabidopsis 2S albumin promoters to the Brazil nut 2S albumin coding sequence and expressing such combinations in tobacco, Arabidopsis, or *Brassica napus* (Altenbach et al. (1989) *Plant Mol. Biol.* 13:513-522; Altenbach et al. (1992) Plant *Mol. Biol.* 18:235-245; De Clercq et al. (1990) *Plant Physiol.* 94:970-979), bean lectin and bean β-phaseolin promoters to

Introns isolated from the maize Adh-1 and Bz-1 genes (Callis et al. (1987) *Genes Dev.* 1:1183-1200), and intron 1 and exon 1 of the maize Shrunken-1 (sh-1) gene (Maas et al. (1991) *Plant Mol. Biol.* 16:199-207) may also be of use to increase expression of introduced genes. Results with the first intron of the maize alcohol dehydrogenase (Adh-1) gene indicate that when this DNA element is placed within the transcriptional unit of a heterologous gene, mRNA levels can be increased by 6.7-fold over normal levels. Similar levels of intron enhancement have been observed using intron 3 of a maize actin gene (Luehrsen, K. R. and Walbot, V. (1991) *Mol. Gen. Genet.* 225:81-93). Enhancement of gene expression by Adh1 intron 6 (Oard et al. (1989) *Plant Cell Rep* 8:156-160) has also been noted. Exon 1 and intron 1 of the maize sh-1 gene have been shown to individually increase expression of reporter genes in maize suspension cultures by 10 and 100-fold, respectively. When used in combination, these elements have been shown to produce up to 1000-fold stimulation of reporter gene expression (Maas et al. (1991) *Plant Mol. Biol.* 16:199-207).

Any 3' non-coding region capable of providing a polyadenylation signal and other regulatory sequences that may be required for proper expression can be used to accomplish the invention. This would include the 3' end from any storage protein such as the 3' end of the 10kd, 15kd, 27kd and alpha zein genes, the 3' end of the bean phaseolin gene, the 3' end of the soybean b-conglycinin gene, the 3' end from viral genes such as the 3' end of the 35S or the 19S cauliflower mosaic virus transcripts, the 3' end from the opine synthesis genes, the 3' ends of ribulose 1,5-bisphosphate carboxylase or chlorophyll a/b binding protein, or 3' end sequences from any source such that the sequence employed provides the necessary regulatory information within its nucleic acid sequence to result in the proper expression of the promoter/coding region combination to which it is operably linked. There are numerous examples in the art that teach the usefulness of different 3' non-coding regions (for example, see Ingelbrecht et al. (1989) *Plant Cell* 1:671-680).

Various methods of introducing a DNA sequence (i.e., of transforming) into eukaryotic cells of higher plants are available to those skilled in the art (see EPO publications 0 295 959 A2 and 0 138 341 A1). Such methods include high-velocity ballistic bombardment with metal particles coated with the nucleic acid constructs (see Klein et al. (1987) *Nature* (London) 327:70-73, and see U.S. Pat. No. 4,945,050), as well as those based on transformation vectors based on the Ti and Ri plasmids of *Agrobacterium spp.*, particularly the binary type of these vectors. Ti-derived vectors transform a wide variety of higher plants, including monocotyledonous and dicotyledonous plants, such as soybean, cotton and rape (Pacciotti et al. (1985)

particular genes. U. S. Pat. Nos. 5,190,931, 5,107,065 and 5,283,323 have taught the feasibility of these techniques, but it is well known that their efficiency is unpredictable. In either case, in order to save time, the person skilled in the art will make multiple genetic constructs containing one or more different parts of the gene to be suppressed, since the art does not teach a method to predict which will be most effective for a particular gene. Furthermore, even the most effective constructs will give an effective suppression phenotype only in a fraction of the individual transgenic lines isolated. For example, WO93/11245 and WO94/11516 teach that when attempting to suppress the expression of fatty acid desaturase genes in canola, actual suppression was obtained in less than 1% of the lines tested. In other species the percentage is somewhat higher, but in no case does the percentage reach 100.

This should not be seen as a limitation on the present invention, but instead as practical matter that is appreciated and anticipated by the person skilled in this art. Accordingly, skilled artisan will develop methods for screening large numbers of transformants. The nature of these screens will generally be chosen on practical grounds, and is not an inherent part of the invention. In the instant case, for example, one can screen by looking for changes in starch phenotype using chromatography to determine relative proportions of amylose to amylopectin, amylopectin branch chain distribution, RVA analysis (as is done in the examples), or other means. One could equally use antibodies specific for the protein encoded by the gene being suppressed, or one could establish assays that specifically measure enzyme activity. A preferred method will be one which allows large numbers of samples to be processed rapidly, since it will be expected that the majority of samples will be negative.

Plants that are identified to have the altered starch fine structure in the grain present unique genetic material which provide advantages over traditional corn lines and known starch mutants. Use of lines with inhibited expression of SBE isoforms in corn breeding provide a dominant trait that can simplify and speed the breeding process. Known starch mutants can be used but they are often recessive and present more complications. Further, the use of antisense or cosuppression to inhibit SBE isoforms leads to variable levels of inhibition due to chromosomal position effects. The resulting variable levels of SBE activities would lead to a wide range of phenotypes that is not possible using traditional mutants which can result in a limited dosage series of a mutant allele in corn endosperm. Additional unique and potentially valuable starch fine structures will result from crossing the newly developed corn lines with inhibited SBE with each other and/or known starch mutants such as *wx* or *ae*.

Cloning sites (NcoI or SmaI) were incorporated into the oligonucleotides to provide antisense orientation of the DNA fragments when inserted into the digested vector pML103 as described below. Amplification was performed in a 100 ml volume in a standard PCR mix consisting of 0.4 mM of each oligonucleotide and 0.3 pM of pBE240 in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% w/v gelatin, 200 mM dGTP, 200 mM dATP, 200 mM dTTP, 200 mM dCTP and 0.025 unit Amplitaq™ DNA polymerase. Reactions were carried out in a Perkin-Elmer Cetus Thermocycler™ for 30 cycles comprising 1 minute at 95°C, 2 minutes at 55°C and 3 minutes at 72°C, with a final 7 minute extension at 72°C after the last cycle. The amplified DNA was digested with restriction enzymes NcoI and SmaI and fractionated on a 0.7% low melting point agarose gel in 40 mM Tris-acetate, pH 8.5, 1 mM EDTA. The appropriate band was excised from the gel, melted at 68°C and combined with a 4.9 kb NcoI-SmaI fragment of the plasmid pML103 (Figure 3). Plasmid pML103 has been deposited under the terms of the Budapest Treaty at ATCC (American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852), and bears the following accession number: ATCC 97366. The DNA segment from pML103 contains a 1.05 kb SalI-NcoI promoter fragment of the maize 27 kD zein gene and a 0.96 kb SmaI-SalI fragment from the 3' end of the maize 10 kD zein gene in the vector pGem9Zf(+) (Promega). Vector and insert DNA were ligated at 15°C overnight, essentially as described (Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning, Cold Spring Harbor Laboratory Press, New York; hereinafter "Maniatis"). The ligated DNA was used to transform *E. coli* XL1-Blue (Epicurian Coli XL-1 Blue™; Stratagene). Bacterial transformants were screened by restriction enzyme digestion of plasmid DNA and limited nucleotide sequence analysis using the dideoxy chain termination method (Sequenase™ DNA Sequencing Kit; U. S. Biochemical). The resulting plasmid construct, pBE44, comprises a chimeric gene encoding, in the 5' to 3' direction, the maize 27 kD zein promoter, a 3' fragment of the corn SBEIIb cDNA, and the 10 kD zein 3' region.

Larger quantities of pBE44 plasmid DNA were prepared by the alkaline lysis method, followed by purification by CsCl density gradient centrifugation.

Transformation of Corn with the 3' Antisense Construct

Immature corn embryos were dissected from developing caryopses derived from crosses of the inbred corn lines H99 and LH132. The embryos were isolated 10 to 11 days after pollination when they were 1.0 to 1.5 mm long. The embryos were placed with the axis-side facing down and in contact with agarose-solidified N6 medium (Chu et al. (1975), *Sci. Sin. Peking* 18:659-668). The embryos were kept in the dark at 27°C.

continued to grow slowly on this medium. After an additional 2 weeks the tissue was transferred to fresh N6 medium containing gluphosinate. After 6 weeks, areas of about 1 cm in diameter of actively growing callus were identified on some of the plates containing the glufosinate-supplemented medium. These calli continued to grow when sub-cultured on the selective medium.

Plants were regenerated from the transgenic callus by first transferring clusters of tissue to N6 medium supplemented with 0.2 mg per liter of 2,4-D. After two weeks the tissue was transferred to regeneration medium (Fromm et al. (1990) *Bio/Technology* 8:833-839). A total of 9 corn plants were regenerated from a single transformation experiment using the pBE44 construct.

Molecular Analysis of Transgenic Corn Plants Containing the 3' Antisense Construct

Total DNA was isolated from leaf tissue of plants regenerated from the transformation experiment using pBE44 essentially as described by Dellaporta et al (Dellaporta et al. (1983) *Plant Mol. Biol. Rep.*1 (4):9). Lyophillized tissue was frozen in liquid nitrogen, ground to a fine powder and suspended in a buffer consisting of 100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 10 mM b-mercaptoethanol and 0.5 M NaCl. Cells were lysed by the addition of SDS to 1% and the DNA precipitated with isopropanol. The dissolved DNA was treated with DNase-free RNase and then re-precipitated with iso-propanol. The isolated DNAs were dissolved in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and stored at -20°C until use.

For Southern blot analysis, 5 mg of isolated DNA was digested with restriction enzyme (10 units/mg DNA) in the appropriate buffer for approximately 6 hrs at 37°C. The restricted DNA was loaded onto a 0.8% agarose gel in Tris-borate-EDTA buffer (Maniatis) and electrophoresed at 40 V overnight. Following denaturation and neutralization, the DNA was transferred to an Immobilon™ membrane (Millipore Corporation) using 10X SSC. The Immobilon™ membrane was pre-hybridized at 65°C in an aqueous buffer system consisting of 6X SSPE, 5X Denhardt's reagent, 0.5% SDS and 100 mg/mL denatured salmon sperm DNA as described (Maniatis). The SBE fragment of pBE44 was labeled by nick translation (BRL Nick Translation Kit) and added to the above buffer supplemented with 5% dextran sulfate at a level of 1-2 x 10^6 cpm/ml. Hybridization was allowed to proceed at 65°C for 18 h. The membrane was sequentially washed with 2X SSC, 0.1% SDS for 15 minutes at room temperature, 1X SSC, 0.1% SDS for 15 minutes at room temperature and 0.1X SSC, 0.5% SDS for 15 minutes at 50°C. Washed membranes were exposed to Dupont Reflection™ film with an intensifying screen at -80°C.

using a paint mixer, and allowed to settle for 30 minutes. Two mL of 100 mM NaCl was sprayed onto the solution, allowed to settle for 30 minutes, and the protein-toluene layer was aspirated off. The toluene wash step was repeated. Twelve mL water was added and shaken in a paint shaker for 45 seconds. This solution was centrifuged for 10 minutes and the water was removed. The water wash was repeated, followed by a final wash with 12 mL of acetone. After shaking and centrifugation steps, the acetone was drained and allowed to evaporate for 1 h. Starch extracts were incubated in a 40°C oven overnight.

Extracted starches were enzymatically debranched as follows. Extracted starches (10 mg) from individual seeds were gelatinized in 2 mL water by heating to 115°C for 0.5 h. Four units of isoamylase (Sigma) in 50 mM NaOAc buffer, pH 4.5, were added to each of the gelatinized starches and placed in a water bath at 45°C for 2.5 h. Enzyme was inactivated by heating samples to 115°C for 5 minutes. Each sample was filtered through a 0.45 micron filter, and placed into individual autosampler vials. Samples were held at 45°C until injection.

Fifty mL of debranched starch sample were injected and run through four columns (3 x 250 Å and 1 x 500 Å ultrahydrogel™; Waters) arranged in series at 45°C and eluted with 50 mM NaOAc at a flow rate of 0.7 mL/min. Sampling interval was 65 minutes. A refractive index detector (Waters), integrator/plotter (Spectra-Physics) and computer were used for sample detection, recording of retention times and chromatogram storage, respectively. Retention times of collected samples were compared to retention times of pullulan standards (380K, 100K, 23.7K, 5.8K, 728 and 180 mw).

Spectra-Physics software was used to make any baseline corrections to the chromatogram including subtraction of a blank chromatogram. Spectra-Physics GPC-PC software was used to enter molecular weights and retention times of pullulan standards. The data were imported to Microsoft Excel for parsing and stripping of all data except molecular weight and area percent of the chromatogram. The remaining data were used to determine branch chain distribution of the amylopectin using Jandel Scientific Peakfit software. A series of six Gaussian curves were fit to the amylopectin portion of the chromatograms as described by Ong et al. ((1994) *Carbohydrate Res.* 260:99-117).

Amylopectin is typically described by its distribution of branch chains in the molecule. The amylopectin molecule is comprised of alternating crystalline and amorphous regions. The crystalline region is where many of the branch points (α–1,6 linkages) occur, while the amorphous region is an area of little to no branching and few branch chains. The type of chain is designated A or B. A chains are unbranched and

the antisense gene relative to control starch. The instant transgenic plants thus demonstrate a unique starch branching phenotype compared to non-transgenic control plants. This data indicates that alteration of corn starch branching enzyme activity by suppressing expression of the corresponding genes encoding starch branching enzymes results in an altered starch phenotype.

R1 kernels from the pBE44 line, 0693, were planted and R2 grain was produced. Individual R2 kernels were analyzed using the same procedure as described above for analysis of R1 kernels. Individual kernels from a negative control line (04659, which has been through the transformation process but does not carry the antisense gene) were included in this set of assays. Table 2 presents the results for R2 kernels. The data represent the percentage difference of the various branches between R2 kernels and kernels from the negative control.

Table 2. Percentage Difference of Branch Chain Distribution of Amylopectin From Starch Isolated From Individual R2 Seed From 3' Antisense SBEIIb Transgenic Corn Line (05985) Compared to Starch Isolated From Negative Control Line (04659).

Starch Source	A + B1	B2	B3	B4+
059852	69	91	132	476
0598510	71	92	129	455

As can be seen, long chains (B3 and B4+) are favored at the expense of shorter chains (A's, B1's and B2's) in the amylopectin derived from R2 kernels possessing the antisense gene relative to control starch (04659). The instant transgenic plant thus demonstrates a unique starch branching phenotype compared to non-transgenic control plants. This data also indicates that the phenotype observed in the R2 seed is stronger than that of the R1 seed (Table 1) which may be due to segregation.

R4 grain (line XAY00681) was produced, harvested and starch was extracted. For starch branch chain distribution and determination of amylose content, starch digestion was modified from that in previous examples slightly as follows. Seven mg of each starch sample was added to a screw cap test tube with 1.1 mL of water. The tubes were heated to 120°C for 30 minutes and then placed in a water bath at 45°C. Debranching solution was made by diluting 50 µL of isoamlyase ($5x10^6$ units/mL, Sigma) per mL of sodium acetate buffer (50 mM, pH 4.5). 40 µL of debranching solution was added to each starch sample and incubated for 3 h at 45°C. Reactions were stopped by heating to 110°C for 5 minutes. Debranched starch samples were lyophilized and redisolved in DMSO for analysis by gel permeation chromatography (GPC). One hundred µL of debranched starch was injected and run through 2 columns (Polymer

21-24	4.72	0.18	3.74	0.05
18-21	6.01	0.03	4.83	0.10
15-18	8.42	0.05	6.18	0.12
13-15	7.24	0.21	5.34	0.11
11-15	6.64	0.17	4.49	0.10
9-11	6.20	0.08	4.54	0.11
7-9	4.48	0.06	3.40	0.07
5-7	3.67	0.07	2.91	0.05

Table 4. Percentage Difference of Branch Chain Distribution of Amylopectin (expressed as A+B1, B2, B3 and B4+) and Amylose Content (% of Total Starch) from Starch Isolated from R4 Grain containing the 3' Antisense Transcript of Corn SBEIIb (XAY00681) as Compared to Control (Dent). DP range is indicated.

A+B1 (5-15)	B2 (15-32)	B3 (32-60)	B4+ (60-150)	Amylose (>150)
83.3	89.0	117.4	184.5	128.4

As can be seen in Tables 3 and 4, the relative amount of amylose increased as did the proportion of longer branches on amylopectin in starch which contained the 3' antisense transcript of corn SBE IIb compared to a dent control.

Functional Analysis of Starch from Lines Homozygous for the 3' Antisense Construct

Kernels of plants of a line (XAT00025), homozygous for the pBE44 construct, were isolated from the progeny of line 05985 in order to obtain sufficient quantities of starch for functionality testing. Starch was extracted from dry mature kernels from line XAT00025, dent, and *ae* corn. For each line 15 g of kernels were weighed into a 50 mL Erlenmeyer flask and steeped in 50 mL of steep solution (same as above) for 18 h at 52°C. The kernels were drained and rinsed with water. The kernels were then homogenized using a 20 mm Polytron probe (Kinematica GmbH; Kriens-Luzern, Switzerland) in 50 mL of cold 50 mM NaCl. The homogenate was filtered through a 72 micron mesh screen. The filtrate was brought up to a total volume of 400 mL with 50 mM NaCl and an equal volume of toluene was added. The mixture was stirred with a magnetic stir bar for 1 h at sufficient speed to completely emulsify the two phases. The emulsion was allowed to separate overnight in a covered beaker. The upper toluene layer was aspirated from the beaker and discarded. The starch slurry remaining in the bottom of the beaker was resuspended, poured into a 250 mL centrifuge bottle and centrifuged 15 minutes at 25,000 RCF. The supernatent was discarded and the starch was washed sequentially with water and acetone by shaking and centrifuging as above.

Thermocycler™ for 30 cycles comprising 1 minute at 95°C, 2 minutes at 55°C and 3 minutes at 72°C, with a final 7 minute extension at 72°C after the last cycle. The amplified DNA was digested with restriction enzymes NcoI and SmaI and fractionated on a 0.7% low melting point agarose gel in 40 mM Tris-acetate, pH 8.5, 1 mM EDTA. The appropriate band was excised from the gel, melted at 68°C and combined with a 4.9 kb NcoI-SmaI fragment of the plasmid pML103 (Figure 3). The DNA segment from pML103 contains a 1.05 kb SalI-NcoI promoter fragment of the maize 27 kD zein gene and a 0.96 kb SmaI-SalI fragment from the 3' end of the maize 10 kD zein gene in the vector pGem9Zf(+) (Promega). Vector and insert DNA was ligated at 15°C overnight, essentially as described (Maniatis). The ligated DNA was used to transform *E. coli* XL1-Blue (Epicurian Coli XL-1 Blue'; Stratagene™). Bacterial transformants were screened by restriction enzyme digestion of plasmid DNA and limited nucleotide sequence analysis using the dideoxy chain termination method (Sequenase™ DNA Sequencing Kit; U. S. Biochemical). The resulting plasmid construct, pBE43, comprises a chimeric gene encoding in the 5' to 3' direction, the maize 27 kD zein promoter, a 5' fragment of the corn SBEIIb gene in antisense orientation, and the 10 kD zein 3' region.

Larger quantities of pBE43 plasmid DNA were prepared by the alkaline lysis method, followed by purification by CsCl density gradient centrifugation.

Transformation of Corn with the 5' Antisense Construct

The 5' antisense construct (pBE43) was introduced into embryogenic corn tissue by the particle bombardment method essentially as described in Example 1. Seven days after bombardment the tissue was transferred to N6 medium that contained gluphosinate (2 mg per liter) and lacked casein or proline. The tissue continued to grow slowly on this medium. After an additional 2 weeks the tissue was transferred to fresh N6 medium containing gluphosinate. After 6 weeks, areas of about 1 cm in diameter of actively growing callus were identified on some of the plates containing the glufosinate-supplemented medium. These calli continued to grow when sub-cultured on the selective medium.

Plants were regenerated from the transgenic callus by first transferring clusters of tissue to N6 medium supplemented with 0.2 mg per liter of 2,4-D. After two weeks the tissue was transferred to regeneration medium (Fromm et al. (1990) *Bio/Technology* 8:833-839). Ninety-nine transgenic plant lines were generated from 2 separate particle bombardment experiments performed with the DNA construct pBE43.

Molecular Analysis of Transformed Corn Plants Containing the 5' Antisense Construct

Southern blot and Northern blot analyses of DNA and RNA from corn plants transformed with the 5' antisense construct (pBE43) were performed as described in

species. This result is consistent with the identity of the 600 base RNA of the antisense transcript of pBE43.

Analysis of Starch from Transformed Corn Plants Containing the 5' Antisense Construct

Starches from individual R1 kernels of plants transformed with pBE43 (the 5' antisense construct for corn SBEIIb) were extracted and analyzed using the procedure described in Example 1. As known to those skilled in the art, the antisense or cosuppression phenomenon is generally not observed in every individual transgenic line. Therefore, individual kernels from multiple lines were examined. No alterations in starch branch chain distribution were observed for the transgenic lines that were screened. It is believed that the number of lines tested was too small to insure finding a plant where an effective antisense event occurred. As described above, the number of plants that must be screened can be unpredictable and large. It is assumed that if a sufficiently large number of individuals were examined such an event would be detected. It may be that this particular configuration is less efficient for suppressing expression of this gene; it is for this reason that multiple constructs were prepared and tested.

EXAMPLE 3

Preparation of Transgenic Corn Expressing a Near Full Length Antisense Transcript of Corn Starch Branching Enzyme IIb

Preparation of the Expression Vector Encoding the Near Full Length Antisense Construct

The construct pBE45 is similar to pBE43 and pBE44 except that the SBEIIb fragment is 2.16 kb and contains the entire 5' untranslated region as well as 2.08 kb of the coding region (SEQ ID NO:8). pBE240 was first digested with EcoRI and then subjected to an end filling reaction with the Klenow fragment of DNA polymerase I (Maniatis). The blunt-ended DNA was fractionated on a low melting point agarose gel and the excised band combined with a 4.9 kb NcoI-SmaI fragment of the plasmid pML103 (Figure 3). The DNA segment from pML103 contains a 1.05 kb SalI-NcoI promoter fragment of the maize 27 kD zein gene and a 0.96 kb SmaI-SalI fragment from the 3' end of the maize 10 kD zein gene in the vector pGem9Zf(+) (Promega). Vector and insert DNA were ligated at 15°C overnight, essentially as described (Maniatis). The ligated DNA was used to transform *E. coli* XL1-Blue (Epicurian Coli XL-1 Blue™; Stratagene). Bacterial transformants were screened for the presence of and the orientation of the added DNA by restriction enzyme digestion with KpnI and limited nucleotide sequence analysis using the dideoxy chain termination method (Sequenase™ DNA Sequencing Kit; U. S. Biochemical). According to this analysis, in pBE45, the SBEIIb fragment is present in inverse orientation relative to the 27 kD zein promoter.

Analysis of Starch from Transformed Corn Plants Containing the Near Full Length Antisense Construct

Starches from individual R1 kernels of plants transformed with pBE45 (the near full length antisense construct for corn SBEIIb) were analyzed using the procedure described in Example 1. As known to those skilled in the art, the antisense phenomenon is generally not observed in every individual transgenic line. Therefore, individual kernels from multiple lines were examined and as expected, some, but not all lines possessed kernels demonstrating an altered starch phenotype. Table 5 presents the results for kernels from a transformed corn line which did show a phenotype. The data represent the percentage difference of the various branches between kernels of the transformed line and kernels from a negative control (line 03376, which has been through the transformation process but does not contain the antisense gene).

Table 5. Percentage Difference of Branch Chain Distribution of Amylopectin from Starch Isolated from Individual Seed from Near Full Length Antisense SBEIIb Transgenic Corn Line (9228) Compared to Starch Isolated from Negative Control Line (03376).

Starch Source	A + B1	B2	B3	B4+
92283	92	97	81	192

As can be seen, long chains (B4+) are favored at the expense of shorter chains (A's and B1's, B2's and B3's) in the starch derived from the corn plant possessing the antisense gene relative to control starch (03376). The instant transgenic plant thus demonstrates a unique starch branching phenotype compared to non-transgenic control plants. This data indicates that alteration of corn starch branching enzyme activity by suppressing expression of the corresponding genes encoding starch branching enzymes results in an altered starch phenotype.

EXAMPLE 4

Preparation of Transgenic Corn Expressing a Near Full Length Sense Transcript of Corn Starch Branching Enzyme IIb

Preparation of the Expression Vector Encoding the Near Full Length Sense Construct

Plasmid pBE96 comprises a 2.09 kb fragment of the SBEIIb cDNA (SEQ ID NO:9) joined in the sense orientation to the 27 kD zein promoter and the 10 kD zein 3' end (Figure 8). The SBEIIb fragment commences at the initiating ATG codon of the coding region and terminates 312 bp 5' of the translation termination codon. pBE240 was subjected to site specific mutagenesis (Sculptor™ Mutagenesis Kit, Amersham) to generate an NcoI site at the ATG start site. The mutagenized plasmid was first digested with EcoRI and then rendered blunt-ended by reaction with Klenow. The DNA

amplify this region of the gene by PCR using corn total DNA as the template. A 571 bp 5' fragment was isolated, sequenced and found to be identical to the cDNA over nucleotides 49 to 188. pBE65 was then used as a starting point in the generation of sense and antisense SBEI constructs including pBE68 and pBE97 described below. In the time since these constructs were made and introduced into corn, a second SBEI sequence became available (Fisher et al. (1995) *Plant Physiol.* 108:1313-1314). The 5' terminal 165 bp of pBE65 showed poor agreement with this sequence as it did with the previous SBEI sequence. As a result of subsequent experiments, it is now concluded that pBE65 contains a 165 bp 5' terminal segment that is not related to SBEI but which presumably arose as an artifact during the cloning of corn cDNA. This region is followed by 2607 bp of SBEI cDNA which encodes 42 amino acids of the SBEI transit peptide, the 760 amino acids of the mature SBEI protein and contains 194 bp of 3' untranslated DNA. The plasmid pBE65 has been deposited under the terms of the Budapest Treaty at the ATCC (American Type Tissue Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852) and bears the following accession number:_____.

Preparation of Expression Vectors Encoding SBEI Antisense Constructs

Since it was not known which portions of the cDNA sequence would be most effective in mediating suppression of SBEI expression, three constructs bearing different SBEI fragments in antisense orientation were made. The chimeric gene of plasmid pBE68 (Figure 9) comprises a 3' fragment of the SBEI cDNA in antisense orientation with respect to the maize 27 kD zein promoter that is located 5' to the SBEI fragment, and the 10 kD zein 3' end that is located 3' to the SBEI fragment. The 373 bp SBEI fragment of this construct (SEQ ID NO:13) was obtained by PCR of pBE65 using the oligonucleotide primer pair BE43 (SEQ ID NO:14) and BE52 (SEQ ID NO:15):

BE43 5'-GAATTCCCGGGCCGAACTCGTTCAAAG-3' (SEQ ID NO:14)

BE52 5'-GAATTCCATGGCGGTGATGAGACACCAGTC-3' (SEQ ID NO:15)

The chimeric gene of pBE69 (Figure 10) is analogous to that of pBE68 except that the SBEI fragment consists of a 5' portion of the SBEI cDNA. The 571 bp fragment of this construct (SEQ ID NO:16) was obtained by amplification of pBE65 using the primer pair BE46 (SEQ ID NO:17) and BE50 (SEQ ID NO:18):

BE46 5'-GAATTCCATGGCCATCTTATGGTTTGCACC-3' (SEQ ID NO:17)

BE50 5'-GAATTCCCGGGCATAGCATAGATATGACGGC-3' (SEQ ID NO:18)

Example 1. Seven days after bombardment, the tissue was transferred to N6 medium that contained gluphosinate (2 mg per liter) and lacked casein or proline. The tissue continued to grow slowly on this medium. After an additional 2 weeks, the tissue was transferred to fresh N6 medium containing gluphosinate. After 6 weeks, areas of about 1 cm in diameter of actively growing callus were identified on some of the plates containing the gluphosinate supplemented medium. These calli continued to grow when sub-cultured on the selective medium.

Plants were regenerated from the transgenic callus by first transferring clusters of tissue to N6 medium supplemented with 0.2 mg per liter of 2,4-D. After two weeks, the tissue was transferred to regeneration medium (Fromm et al. (1990) *Bio/Technology* 8:833-839). Nine transgenic plant lines were regenerated from particle bombardment experiments performed with the DNA construct pBE68, 20 transgenic lines were regenerated from particle bombardments performed with the DNA construct pBE69 and 9 transgenic lines were regenerated from particle bombardmdent experiments performed with the DNA construct pBE72.

Molecular Analysis of Transgenic Corn Plants Containing the SBEI Antisense Constructs

Total DNA was isolated from leaf tissue of transgenic plants essentially as described in Example 1. For Southern blot analysis of pBE68, pBE69 and pBE72 transformants, 10 mg of isolated DNA was digested with the restriction enzyme XbaI at 37°C for 6 hrs in the buffer supplied by the manufacturer. The restricted DNAs were electrophoresed at 40 volts overnight on a 0.8 % agarose gel in Tris-phosphate-EDTA buffer (Maniatis) and transferred to Immobilon™ membranes. The blots were pre-hybridized, hybridized with nick translated pBE65 insert, and washed as described in Example 1.

Total RNA was isolated from developing (20-22 DAP) kernels of transgenic plants and Northern blots were prepared as described in Example 1. Blots were probed with nick translated pBE65 insert DNA and subsequently washed according to the regimen outlined in Example 1.

Of the 9 transgenic plant lines that were regenerated from particle bombardments with the pBE68 construct, 5 were identified by Southern blot analysis to contain the trait gene. Northern blot analysis showed variable levels of the 2.7 kb SBEI mRNA in 4 of the Southern positive lines. In addition, 2 of these lines contained a 400 base transcript that presumably corresponds to the antisense RNA specified by the chimeric gene of pBE68. Of the 20 transgenic plant lines that were generated from bombardments with pBE69, 8 were found to contain pBE69 DNA. RNA isolated from two of the pBE69 transgenic plant lines showed the presence of the 600 base antisense transcript. Of the 9

Table 6. The Percentage of Total Chromatographic Area within Given Degree of Polymerization (DP) Ranges for Starch Derived from R4 Kernels Containing the 5' Antisense Transcript of Corn SBE I (XAY01414) and Dent Starch (control). Average of 12 individual seed and standard errors of the mean (SE) are provided.

	Dent Starch		XAY01414	
DP range	Average	SE	Average	SE
>5k	5.45	0.14	5.92	0.14
3-5k	2.62	0.05	2.58	0.04
1.8-3k	3.03	0.04	2.95	0.08
1.2-1.8k	2.49	0.05	2.66	0.03
0.9-1.2k	1.92	0.04	2.01	0.04
600-900	2.86	0.03	2.94	0.06
400-600	2.78	0.05	3.07	0.04
250-400	2.83	0.05	3.23	0.04
150-250	2.43	0.04	2.97	0.05
90-150	2.38	0.04	3.61	0.06
60-90	4.04	0.08	5.72	0.15
48-60	4.08	0.07	4.94	0.10
40-48	3.95	0.09	4.86	0.04
32-40	4.52	0.13	5.59	0.14
28-32	3.45	0.12	3.58	0.17
24-28	3.69	0.17	4.40	0.08
21-24	4.72	0.18	4.06	0.18
18-21	6.01	0.03	5.64	0.23
15-18	8.42	0.05	6.17	0.16
13-15	7.24	0.21	5.92	0.28
11-15	6.64	0.17	5.33	0.15
9-11	6.20	0.08	4.71	0.13
7-9	4.48	0.06	3.58	0.09
5-7	3.67	0.07	3.44	0.06

21-24	4.72	0.18	4.67	0.18
18-21	6.01	0.03	5.40	0.12
15-18	8.42	0.05	6.64	0.16
13-15	7.24	0.21	5.73	0.22
11-15	6.64	0.17	5.23	0.11
9-11	6.20	0.08	5.27	0.10
7-9	4.48	0.06	4.08	0.09
5-7	3.67	0.07	3.31	0.10

Table 9. Percentage Difference of Branch Chain Distribution of Amylopectin (expressed as A+B1, B2, B3 and B4+) and Amylose Content (% of Total Starch) from Starch Isolated from R4 Kernels containing the 3' Antisense Transcript of Corn SBE I (XAY00013) as Compared to Control (Dent). DP range is indicated.

A+B1 (5-15)	B2 (15-32)	B3 (32-60)	B4+ (60-150)	Amylose (>150)
85.6	95.9	123.1	135.1	106.0

Like the XAY01414 line, the line transformed with the pBE68 construct has alterations in both the amylose and amylopectin fractions of the starch. Amylose content is increased relative to the control and longer chains (B4+ and B3) are increased in the amylopectin. The majority of the increase in amylose content is due to an increse in the Amylose of DP greater than 5000.

The instant transgenic plants thus demonstrate a unique starch branching pattern compared to the control plants. This data indicates that alteration of corn starch branching enzyme activity by suppressing expression of the corresponding genes encoding starch branching enzymes results in an altered starch phenotype.

EXAMPLE 6

Preparation of Transgenic Corn Expressing Sense Transcripts of Corn Starch Branching Enzyme I

Preparation of the Expression Vector Encoding the Near Full Length Sense Construct

Plasmid pBE97 comprises a 1.87 kb fragment of the SBEI cDNA of pBE65 (SEQ ID NO:20) joined in the sense orientation to the 27 kD zein promoter and the 10 kD zein 3' end (Figure 13). The SBEI fragment encompasses nucleotides 55 through 1919 of the cDNA clone pBE65 and thus contains 117 bp of unknown sequence preceding the remaining 1748 bp of SBEI coding region DNA. This DNA fragment was generated by PCR-mediated site specific mutagenesis to introduce an NcoI site at nucleotide position 53 of the pBE65 sequence. The appropriate nucleotide primers were combined with pBE65 template DNA in a standard PCR reaction defined in Example 1.

pBE85. pBE85 was subjected to partial digestion with PvuII and the 4.7 kb 10 kD zein-SBEI-10 kD zein fragment was inserted into PvuII digested pKS17 (Example 4). The resultant construct designated pBE98, contains 110 bp of unidentified sequence at the 5' end of SBEI cDNA segment. The correct 5' sequence of the SBEI cDNA was obtained by PCR using oligonucleotides BE101 (SEQ ID NO:22) and BB3 (SEQ ID NO:23):

BE101 5'-AACTGCAGAAGGATCCCATGGTGTGCCTCGTGTCGCCC-3' (SEQ ID NO:22)

BB3 5'-GGATGCTTAAATGTGTACC-3' (SEQ ID NO:23)

and lambda DNA prepared from plate lysates of a 19 DAP corn endosperm cDNA library (Stratagene) as the template. The 748 bp PCR product was digested with NcoI and SstI to yield a 673 bp fragment. This DNA segment was exchanged with the corresponding region in pBE98 to give pBE110. The construct pBE110 is 7203 bp in length and consists of a 2565 bp segment of SBEI cDNA (SEQ ID NO:24) that includes the entire 823 amino acids of the SBEI coding region and 96 bp of 3' untranslated DNA (Figure 14). The SBEI DNA fragment is preceded by the promoter region of the maize 10 kD zein gene and is followed by the 3' end of the maize 10 kD zein gene.

The truncated sense SBEI construct pBE111 was generated by assembling a shortened SBEI coding region fragment in the vector pBC24. pBC24 is a pSK+ derivative in which the XbaI site has been blunted by reaction with the Klenow fragment of DNA polymerase and ligated to NcoI linkers. pBC24 thus lacks the XbaI site and contains a unique NcoI site in the polylinker region. The 5' SBEI fragment described above was digested with the restriction enzymes NcoI and BamHI and the 694 bp fragment was cloned into NcoI-BamHI digested pBC24. This intermediate was then digested with BamHI and SmaI and ligated to the 1874 bp BamHI-SmaI fragment of pBE83 to yield pBE112. pBE112 was digested with BstEII, reacted with Klenow and then subjected to partial digestion with NcoI. The liberated 1809 bp fragment was cloned into NcoI-partial SmaI digested pBT752. The vector, pBT752 is a derivative of pKS17 described in Example 4 which contains a 27 kD zein-maize high sulfur zein-10 kD zein chimeric gene and lacks the NcoI site at the translational start site of the hygromycin phosphotransferase gene. Analytical digests of the resultant transformants in NovaBlue (Novagen) cells revealed that the 10 kD zein 3' end was removed as a SmaI fragment during the cloning procedure. This 963 bp SmaI segment was thus isolated from pBT752 and inserted into a blunted HindIII site that is located just downstream from BstEII/SmaI junction in the intermediate plasmid, pBE110.5. Transformants were screened by digestion with DraI in order to determine the orientation of the 3' end fragment relative to the chimeric SBEI gene. From this analysis, pBE111 was identified.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: E. I. DU PONT DE NEMOURS AND COMPANY
(B) STREET: 1007 MARKET STREET
(C) CITY: WILMINGTON
(D) STATE: DELAWARE
(E) COUNTRY: UNITED STATES OF AMERICA
(F) POSTAL CODE (ZIP): 19898
(G) TELEPHONE: 302-992-4927
(H) TELEFAX: 302-773-0164
(I) TELEX: 6717325

(ii) TITLE OF INVENTION: NOVEL STARCHES VIA MODIFICATION OF EXPRESSION OF STARCH BIOSYNTHESIS ENZYME GENES

(iii) NUMBER OF SEQUENCES: 25

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: DISKETTE, 3.50 INCH
(B) COMPUTER: IBM PC COMPATIBLE
(C) OPERATING SYSTEM: WINDOWS 3.1
(D) SOFTWARE: MICROSOFT WORD 6.0A

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 06/009,113
(B) FILING DATE: DECEMBER 20, 1995

(vii) ATTORNEY/AGENT INFORMATION:
(A) NAME: BRUCE W. MORRISSEY
(B) REGISTRATION NUMBER: 30,663
(C) REFERENCE/DOCKET NUMBER: BB-1066

```
GCA TTG GTG GGT GAC TTC AAC AAC TGG GAT CCA AAT GCA GAT CGT ATG      687
Ala Leu Val Gly Asp Phe Asn Asn Trp Asp Pro Asn Ala Asp Arg Met
        190                 195                 200

AGC AAA AAT GAG TTT GGT GTT TGG GAA ATT TTT CTG CCT AAC AAT GCA      735
Ser Lys Asn Glu Phe Gly Val Trp Glu Ile Phe Leu Pro Asn Asn Ala
    205                 210                 215

GAT GGT ACA TCA CCT ATT CCT CAT GGA TCT CGT GTA AAG GTG AGA ATG      783
Asp Gly Thr Ser Pro Ile Pro His Gly Ser Arg Val Lys Val Arg Met
220                 225                 230                 235

GAT ACT CCA TCA GGG ATA AAG GAT TCA ATT CCA GCC TGG ATC AAG TAC      831
Asp Thr Pro Ser Gly Ile Lys Asp Ser Ile Pro Ala Trp Ile Lys Tyr
                240                 245                 250

TCA GTG CAG GCC CCA GGA GAA ATA CCA TAT GAT GGG ATT TAT TAT GAT      879
Ser Val Gln Ala Pro Gly Glu Ile Pro Tyr Asp Gly Ile Tyr Tyr Asp
            255                 260                 265

CCT CCT GAA GAG GTA AAG TAT GTG TTC AGG CAT GCG CAA CCT AAA CGA      927
Pro Pro Glu Glu Val Lys Tyr Val Phe Arg His Ala Gln Pro Lys Arg
        270                 275                 280

CCA AAA TCA TTG CGG ATA TAT GAA ACA CAT GTC GGA ATG AGT AGC CCG      975
Pro Lys Ser Leu Arg Ile Tyr Glu Thr His Val Gly Met Ser Ser Pro
    285                 290                 295

GAA CCG AAG ATA AAC ACA TAT GTA AAC TTT AGG GAT GAA GTC CTC CCA     1023
Glu Pro Lys Ile Asn Thr Tyr Val Asn Phe Arg Asp Glu Val Leu Pro
300                 305                 310                 315

AGA ATA AAA AAA CTT GGA TAC AAT GCA GTG CAA ATA ATG GCA ATC CAA     1071
Arg Ile Lys Lys Leu Gly Tyr Asn Ala Val Gln Ile Met Ala Ile Gln
                320                 325                 330

GAG CAC TCA TAT TAT GGA AGC TTT GGA TAC CAT GTA ACT AAT TTT TTT     1119
Glu His Ser Tyr Tyr Gly Ser Phe Gly Tyr His Val Thr Asn Phe Phe
            335                 340                 345

GCG CCA AGT AGT CGT TTT GGT ACC CCA GAA GAT TTG AAG TCT TTG ATT     1167
Ala Pro Ser Ser Arg Phe Gly Thr Pro Glu Asp Leu Lys Ser Leu Ile
        350                 355                 360

GAT AGA GCA CAT GAG CTT GGT TTG CTA GTT CTC ATG GAT GTG GTT CAT     1215
Asp Arg Ala His Glu Leu Gly Leu Leu Val Leu Met Asp Val Val His
    365                 370                 375

AGT CAT GCG TCA AGT AAT ACT CTG GAT GGG TTG AAT GGT TTT GAT GGT     1263
Ser His Ala Ser Ser Asn Thr Leu Asp Gly Leu Asn Gly Phe Asp Gly
380                 385                 390                 395

ACA GAT ACA CAT TAC TTT CAC AGT GGT CCA CGT GGC CAT CAC TGG ATG     1311
Thr Asp Thr His Tyr Phe His Ser Gly Pro Arg Gly His His Trp Met
                400                 405                 410

TGG GAT TCT CGC CTA TTT AAC TAT GGG AAC TGG GAA GTT TTA AGA TTT     1359
Trp Asp Ser Arg Leu Phe Asn Tyr Gly Asn Trp Glu Val Leu Arg Phe
            415                 420                 425

CTT CTC TCC AAT GCT AGA TGG TGG CTC GAG GAA TAT AAG TTT GAT GGT     1407
Leu Leu Ser Asn Ala Arg Trp Trp Leu Glu Glu Tyr Lys Phe Asp Gly
        430                 435                 440
```

```
GAT CAC CAG TAT ATT TCC CGG AAA CAT GAG GAG GAT AAG GTG ATT GTG   2223
Asp His Gln Tyr Ile Ser Arg Lys His Glu Glu Asp Lys Val Ile Val
700                 705                 710                 715

TTC GAA AAG GGA GAT TTG GTA TTT GTG TTC AAC TTC CAC TGC AAC AAC   2271
Phe Glu Lys Gly Asp Leu Val Phe Val Phe Asn Phe His Cys Asn Asn
                720                 725                 730

AGC TAT TTT GAC TAC CGT ATT GGT TGT CGA AAG CCT GGG GTG TAT AAG   2319
Ser Tyr Phe Asp Tyr Arg Ile Gly Cys Arg Lys Pro Gly Val Tyr Lys
            735                 740                 745

GTG GTC TTG GAC TCC GAC GCT GGA CTA TTT GGT GGA TTT AGC AGG ATC   2367
Val Val Leu Asp Ser Asp Ala Gly Leu Phe Gly Gly Phe Ser Arg Ile
        750                 755                 760

CAT CAC GCA GCC GAG CAC TTC ACC GCC GAC TGT TCG CAT GAT AAT AGG   2415
His His Ala Ala Glu His Phe Thr Ala Asp Cys Ser His Asp Asn Arg
    765                 770                 775

CCA TAT TCA TCC TCG GTT TAT ACA CCA AGC AGA ACA TGT GTC GTC TAT   2463
Pro Tyr Ser Ser Ser Val Tyr Thr Pro Ser Arg Thr Cys Val Val Tyr
780                 785                 790                 795

GCT CCA GTG GAG T GATAGCGGGG TACTCGTTGC TGCGCGGCAT GTGTGGGGCT     2516
Ala Pro Val Glu

GTCGATGTGA GGAAAAACCT TCTTCCAAAA CCGGCAGATG CATGCATGCA TGCTACAATA 2576

AGGTTCTGAT ACTTTAATCG ATGCTGGAAA GCCCATGCAT CTCGCTGCGT TGTCCTCTCT 2636

ATATATATAA GACCTTCAAG GTGTCAATT                                   2665
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 414 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
GACACCTTGA AGGTCTTATA TATATAGAGA GGACAACGCA GCGAGATGCA TGGGCTTTCC   60

AGCATCGATT AAAGTATCAG AACCTTATTG TAGCATGCAT GCATGCATCT GCCGGTTTTG  120

GAAGAAGGTT TTTCCTCACA TCGACAGCCC CACACATGCC GCGCAGCAAC GAGTACCCCG  180

CTATCACTCC ACTGGAGCAT AGACGACACA TGTTCTGCTT GGTGTATAAA CCGAGGATGA  240

ATATGGCCTA TTATCATGCG AACAGTCGGC GGTGAAGTGC TCGGCTGCGT GATGGATCCT  300

GCTAAATCCA CCAAATAGTC CAGCGTCGGA GTCCAAGACC ACCTTATACA CCCCAGGCTT  360

TCGACAACCA ATACGGTAGT CAAAATAGCT GTTGTTGCAG TGGAAGTTGA ACAC        414
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GAATTCCCGG GACCCGGATT TCGCTCTT 28

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GAATTCCATG GTCTATAGAG GCTGTACCG 29

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2165 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AATTCATATT TTTGCTCAAG ATGTTGCATT GCCTGATCAA ACTCTTGCAT ACCATGATAC 60

CTAAGATAGT CTGCATCACC CAGGTCAAAT CTTCGACGAC ATTTGTCATA ACTGTTGTTA 120

TTCCCTGGAA TAAACTTACC ACTTGGAAGT CTTTGCGGAC CTCTTGGAAA ATCTATCCAT 180

TCAGGATGTC CAAACTCATT TCCCATGAAA TTAAGATAGC CCTCTCCTCC TAAACCCATT 240

GTGATAAGTC TAATCATCTT ATGTAATGCT ATCCCACGAT CAATGGTAGG AGTTGAAGGT 300

CTATCGAGGG CCATGAAATC ATACATATCC TTGTCCATCA ACCAAAACGC AATAGTCTTG 360

TCGCCGACTA ATGCTTGATC ATGACTTTCA GCATAAGTTA CACACTTCTC TAACCACCTC 420

CTATTTGTCA GTGTGTGCAC AATATCACCC ATCTTCCAAG TTTCATCACT TTGCTTGAGA 480

AGGTCAATCC ATTTGTCAGC CACAGCCATA TGCATCCGAT AGTCAAAACC TACCCCACCA 540

TCGTGAACAG GAAGGGCAAA TGTAGGCATT CCACTAACAT CTTCACCAAT GGTTACAGCC 600

TCAGGATAAA GTCCATGAAT TAGATCATTT ACCAGCATCA AGTAAACCAC TGCATCTACA 660

TCGGTGGCAA AGCCAAAATA CTCATTGAAG TTCCCCGTAA ATGTTACTTG TAATCCGTGG 720

TGAGTGTACA TCATGGAGGT CACACCATCA AAACGGAAAC CATCAAACTT ATATTCCTCG 780

AGCCACCATC TAGCATTGGA GAGAAGAAAT CTTAAAACTT CCCAGTTCCC ATAGTTAAAT 840

AGGCGAGAAT CCCACATCCA GTGATGGCCA CGTGGACCAC TGTGAAAGTA ATGTGTATCT 900

GTACCATCAA AACCATTCAA CCCATCCAGA GTATTACTTG ACGCATGACT ATGAACCACA 960

TCCATGAGAA CTAGCAAACC AAGCTCATGT GCTCTATCAA TCAAAGACTT CAAATCTTCT 1020

GGGGTACCAA AACGACTACT TGGCGCAAAA AAATTAGTTA CATGGTATCC AAAGCTTCCA 1080

CTCTATAGAA GAATCCGTTC AGACATTGAT GAACATGAAG GAGGCTTGGA AGCCTTCTCC 480

CGTAGTTATG AGAAGTTTGG ATTTAATGCC AGCGCGGAAG GTATCACATA TCGAGAATGG 540

GCTCCTGGAG CATTTTCTGC AGCATTGGTG GGTGACTTCA ACAACTGGGA TCCAAATGCA 600

GATCGTATGA GCAAAAATGA GTTTGGTGTT TGGGAAATTT TTCTGCCTAA CAATGCAGAT 660

GGTACATCAC CTATTCCTCA TGGATCTCGT GTAAAGGTGA GAATGGATAC TCCATCAGGG 720

ATAAAGGATT CAATTCCAGC CTGGATCAAG TACTCAGTGC AGGCCCCAGG AGAAATACCA 780

TATGATGGGA TTTATTATGA TCCTCCTGAA GAGGTAAAGT ATGTGTTCAG GCATGCGCAA 840

CCTAAACGAC CAAAATCATT GCGGATATAT GAAACACATG TCGGAATGAG TAGCCCGGAA 900

CCGAAGATAA ACACATATGT AAACTTTAGG GATGAAGTCC TCCCAAGAAT AAAAAAACTT 960

GGATACAATG CAGTGCAAAT AATGGCAATC CAAGAGCACT CATATTATGG AAGCTTTGGA 1020

TACCATGTAA CTAATTTTTT TGCGCCAAGT AGTCGTTTTG GTACCCCAGA AGATTTGAAG 1080

TCTTTGATTG ATAGAGCACA TGAGCTTGGT TTGCTAGTTC TCATGGATGT GGTTCATAGT 1140

CATGCGTCAA GTAATACTCT GGATGGGTTG AATGGTTTTG ATGGTACAGA TACACATTAC 1200

TTTCACAGTG GTCCACGTGG CCATCACTGG ATGTGGGATT CTCGCCTATT TAACTATGGG 1260

AACTGGGAAG TTTTAAGATT TCTTCTCTCC AATGCTAGAT GGTGGCTCGA GGAATATAAG 1320

TTTGATGGTT TCCGTTTTGA TGGTGTGACC TCCATGATGT ACACTCACCA CGGATTACAA 1380

GTAACATTTA CGGGGAACTT CAATGAGTAT TTTGGCTTTG CCACCGATGT AGATGCAGTG 1440

GTTTACTTGA TGCTGGTAAA TGATCTAATT CATGGACTTT ATCCTGAGGC TGTAACCATT 1500

GGTGAAGATG TTAGTGGAAT GCCTACATTT GCCCTTCCTG TTCACGATGG TGGGGTAGGT 1560

TTTGACTATC GGATGCATAT GGCTGTGGCT GACAAATGGA TTGACCTTCT CAAGCAAAGT 1620

GATGAAACTT GGAAGATGGG TGATATTGTG CACACACTGA CAAATAGGAG GTGGTTAGAG 1680

AAGTGTGTAA CTTATGCTGA AAGTCATGAT CAAGCATTAG TCGGCGACAA GACTATTGCG 1740

TTTTGGTTGA TGGACAAGGA TATGTATGAT TTCATGGCCC TCGATAGACC TTCAACTCCT 1800

ACCATTGATC GTGGGATAGC ATTACATAAG ATGATTAGAC TTATCACAAT GGGTTTAGGA 1860

GGAGAGGGCT ATCTTAATTT CATGGGAAAT GAGTTTGGAC ATCCTGAATG GATAGATTTT 1920

CCAAGAGGTC CGCAAAGACT TCCAAGTGGT AAGTTTATTC CAGGGAATAA CAACAGTTAT 1980

GACAAATGTC GTCGAAGATT TGACCTGGGT GATGCAGACT ATCTTAGGTA TCATGGTATG 2040

CAAGAGTTTG ATCAGGCAAT GCAACATCTT GAGCAAAAAT ATGAATT 2087

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

```
GAT GTC GAC CAT CTC CCC ATA TAC GAC CTG GAC CCC AAG CTG GAG ATA      393
Asp Val Asp His Leu Pro Ile Tyr Asp Leu Asp Pro Lys Leu Glu Ile
100                 105                 110                 115

TTC AAG GAC CAT TTC AGG TAC CGG ATG AAA AGA TTC CTA GAG CAG AAA      441
Phe Lys Asp His Phe Arg Tyr Arg Met Lys Arg Phe Leu Glu Gln Lys
                120                 125                 130

GGA TCA ATT GAA GAA AAT GAG GGA AGT CTT GAA TCT TTT TCT AAA GGC      489
Gly Ser Ile Glu Glu Asn Glu Gly Ser Leu Glu Ser Phe Ser Lys Gly
            135                 140                 145

TAT TTG AAA TTT GGG ATT AAT ACA AAT GAG GAT GGA ACT GTA TAT CGT      537
Tyr Leu Lys Phe Gly Ile Asn Thr Asn Glu Asp Gly Thr Val Tyr Arg
        150                 155                 160

GAA TGG GCA CCT GCT GCG CAG GAG GCA GAG CTT ATT GGT GAC TTC AAT      585
Glu Trp Ala Pro Ala Ala Gln Glu Ala Glu Leu Ile Gly Asp Phe Asn
    165                 170                 175

GAC TGG AAT GGT GCA AAC CAT AAG ATG GAG AAG GAT AAA TTT GGT GTT      633
Asp Trp Asn Gly Ala Asn His Lys Met Glu Lys Asp Lys Phe Gly Val
180                 185                 190                 195

TGG TCG ATC AAA ATT GAC CAT GTC AAA GGG AAA CCT GCC ATC CCT CAC      681
Trp Ser Ile Lys Ile Asp His Val Lys Gly Lys Pro Ala Ile Pro His
                200                 205                 210

AAT TCC AAG GTT AAA TTT CGC TTT CTA CAT GGT GGA GTA TGG GTT GAT      729
Asn Ser Lys Val Lys Phe Arg Phe Leu His Gly Gly Val Trp Val Asp
            215                 220                 225

CGT ATT CCA GCA TTG ATT CGT TAT GCG ACT GTT GAT GCC TCT AAA TTT      777
Arg Ile Pro Ala Leu Ile Arg Tyr Ala Thr Val Asp Ala Ser Lys Phe
        230                 235                 240

GGA GCT CCC TAT GAT GGT GTT CAT TGG GAT CCT CCT GCT TCT GAA AGG      825
Gly Ala Pro Tyr Asp Gly Val His Trp Asp Pro Pro Ala Ser Glu Arg
    245                 250                 255

TAC ACA TTT AAG CAT CCT CGG CCT TCA AAG CCT GCT GCT CCA CGT ATC      873
Tyr Thr Phe Lys His Pro Arg Pro Ser Lys Pro Ala Ala Pro Arg Ile
260                 265                 270                 275

TAT GAA GCC CAT GTA GGT ATG AGT GGT GAA AAG CCA GCA GTA AGC ACA      921
Tyr Glu Ala His Val Gly Met Ser Gly Glu Lys Pro Ala Val Ser Thr
                280                 285                 290

TAT AGG GAA TTT GCA GAC AAT GTG TTG CCA CGC ATA CGA GCA AAT AAC      969
Tyr Arg Glu Phe Ala Asp Asn Val Leu Pro Arg Ile Arg Ala Asn Asn
            295                 300                 305

TAC AAC ACA GTT CAG TTG ATG GCA GTT ATG GAG CAT TCG TAC TAT GCT     1017
Tyr Asn Thr Val Gln Leu Met Ala Val Met Glu His Ser Tyr Tyr Ala
        310                 315                 320

TCT TTC GGG TAC CAT GTG ACA AAT TTC TTT GCG GTT AGC AGC AGA TCA     1065
Ser Phe Gly Tyr His Val Thr Asn Phe Phe Ala Val Ser Ser Arg Ser
    325                 330                 335
```

```
ACT GGC ATG TCA GAC TTG CAG CCT GCT TCA CCT ACA ATT GAT CGA GGG      1833
Thr Gly Met Ser Asp Leu Gln Pro Ala Ser Pro Thr Ile Asp Arg Gly
580                 585                 590                 595

ATT GCA CTC CAA AAG ATG ATT CAC TTC ATC ACA ATG GCC CTT GGA GGT      1881
Ile Ala Leu Gln Lys Met Ile His Phe Ile Thr Met Ala Leu Gly Gly
                600                 605                 610

GAT GGC TAC TTG AAT TTT ATG GGA AAT GAG TTT GGT CAC CCA GAA TGG      1929
Asp Gly Tyr Leu Asn Phe Met Gly Asn Glu Phe Gly His Pro Glu Trp
            615                 620                 625

ATT GAC TTT CCA AGA GAA GGG AAC AAC TGG AGC TAT GAT AAA TGC AGA      1977
Ile Asp Phe Pro Arg Glu Gly Asn Asn Trp Ser Tyr Asp Lys Cys Arg
        630                 635                 640

CGA CAG TGG AGC CTT GTG GAC ACT GAT CAC TTG CGG TAC AAG TAC ATG      2025
Arg Gln Trp Ser Leu Val Asp Thr Asp His Leu Arg Tyr Lys Tyr Met
    645                 650                 655

AAT GCG TTT GAC CAA GCG ATG AAT GCG CTC GAT GAG AGA TTT TCC TTC      2073
Asn Ala Phe Asp Gln Ala Met Asn Ala Leu Asp Glu Arg Phe Ser Phe
660                 665                 670                 675

CTT TCG TCG TCA AAG CAG ATC GTC AGC GAC ATG AAC GAT GAG GAA AAG      2121
Leu Ser Ser Ser Lys Gln Ile Val Ser Asp Met Asn Asp Glu Glu Lys
                680                 685                 690

GTT ATT GTC TTT GAA CGT GGA GAT TTA GTT TTT GTT TTC AAT TTC CAT      2169
Val Ile Val Phe Glu Arg Gly Asp Leu Val Phe Val Phe Asn Phe His
            695                 700                 705

CCC AAG AAA ACT TAC GAG GGC TAC AAA GTG GGA TGC GAT TTG CCT GGG      2217
Pro Lys Lys Thr Tyr Glu Gly Tyr Lys Val Gly Cys Asp Leu Pro Gly
        710                 715                 720

AAA TAC AGA GTA GCC CTG GAC TCT GAT GCT CTG GTC TTC GGT GGA CAT      2265
Lys Tyr Arg Val Ala Leu Asp Ser Asp Ala Leu Val Phe Gly Gly His
    725                 730                 735

GGA AGA GTT GGC CAC GAC GTG GAT CAC TTC ACG TCG CCT GAA GGG GTG      2313
Gly Arg Val Gly His Asp Val Asp His Phe Thr Ser Pro Glu Gly Val
740                 745                 750                 755

CCA GGG GTG CCC GAA ACG AAC TTC AAC AAC CGG CCG AAC TCG TTC AAA      2361
Pro Gly Val Pro Glu Thr Asn Phe Asn Asn Arg Pro Asn Ser Phe Lys
                760                 765                 770

GTC CTT TCT CCG CCC CGC ACC TGT GTG GCT TAT TAC CGT GTA GAC GAA      2409
Val Leu Ser Pro Pro Arg Thr Cys Val Ala Tyr Tyr Arg Val Asp Glu
            775                 780                 785

GCA GGG GCT GGA CGA CGT CTT CAC GCG AAA CGA GAG ACA GGA AAG ACG      2457
Ala Gly Ala Gly Arg Arg Leu His Ala Lys Arg Glu Thr Gly Lys Thr
        790                 795                 800

TCT CCA GCA GAG AGC ATC GAC GTC AAA GCT TCC AGA GCT AGT AGC AAA      2505
Ser Pro Ala Glu Ser Ile Asp Val Lys Ala Ser Arg Ala Ser Ser Lys
    805                 810                 815
```

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GAATTCCATG GCGGTGATGA GACACCAGTC 30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 571 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CCATCTTATG GTTTGCACCA TTCCAGTCAT TGAAGTCACC AATAAGCTCT GCCTCCTGCG 60

CAGCAGGTGC CCATTCACGA TATACAGTTC CATCCTCATT TGTATTAATC CCAAATTTCA 120

AATAGCCTTT AGAAAAAGAT TCAAGACCTT CCCTCATTTT CTTCAATTGA TCCTTTCTGC 180

TCTAGGAATC TTTTCATCCG GTACCTGAAA TGGTCCTTGA ATATCTCCAG CTTGGGGTCC 240

AGGTCGTATA TGGGGAGATG GTCGACATCG CCTTTGGCAG TTGCCATAGT TTTATCTTCT 300

TGCACAGTAG CTGCAGTGGC GAATTTGCTC TTGACCTTCC GCACCCCTGA CCGGCGAGCC 360

TTGCACTGGA CAGACAACAC ACTCAGGCGC ACATTGCCGC CACCCGCGAT CCCCGGCGGT 420

GCCGCCCGAT CAGCATGCGA GCAAGAGCGA CGATGGCCTT GGAGTCGTCG TCGGCCGGCT 480

TGGCGGGCGA CGGTGGCAAC GGCACGACGG CCTTCTCCTC GGCGATGTCC TTGGCGGCCT 540

CCACCTCCGC CGCCGTCATA TCTATGCTAT G 571

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GAATTCCATG GCCATCTTAT GGTTTGCACC 30

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

TACCTCCCAG TTAGCATAGT TGAACAGCCG ACTATCCCAA AGTTTATGAT AACCTCTATC 1260

TCCCGCATGA AAATAGGACT CTTGGGTGCT TTGTCCAACA TCATAGCCAT TTAAACCATC 1320

TGTGACATTA TTACTTGCAT GGCTATGGAC AACATCCATC AGAACTCGCA AACCCAAACT 1380

GTGTGCCTTA TCAACAAGAT ATTTGAGGTC CTCTGGTGTG CCTGATCTGC TGCTAACCGC 1440

AAAGAAATTT GTCACATGGT ACCCGAAAGA AGCATAGTAC GAATGCTCCA TAACTGCCAT 1500

CAACTGAACT GTGTTGTAGT TATTTGCTCG TATGCGTGGC AACACATTGT CTGCAAATTC 1560

CCTATATGTG CTTACTGCTG GCTTTTCACC ACTCATACCT ACATGGGCTT CATAGATACG 1620

TGGAGCAGCA GGCTTTGAAG GCCGAGGATG CTTAAATGTG TACCTTTCAG AAGCAGGAGG 1680

ATCCCAATGA ACACCATCAT AGGGAGCTCC AAATTTAGAG GCATCAACAG TCGCATAACG 1740

AATCAATGCT GGAATACGAT CAACCCATAC TCCACCATGT AGAAAGCGAA ATTTAACCTT 1800

GGAATTGTGA GGGATGGCAG GTTTCCCTTT GACATGGTCA ATTTTGATCG ACCAAACACC 1860

AAATTTATCC TTCTCCATCT TATGGTTTGC ACCATTCCAG TCATTGAAGT CACCAATAAG 1920

CTCTGCCTCC TGCGCAGCAG GTGCCCATTC ACGATATACA GTTCCATCCT CATTTGTATT 1980

AATCCCAAAT TTCAAATAGC CTTTAGAAAA AGATTCAAGA CTTCCCTCAT TTTCTTCAAT 2040

TGATCCTTTC TGCTCTAGGA ATCTTTTCAT CCGGTACCTG AAATGGTCCT TGAATATCTC 2100

CAGCTTGGGG TCCAGGTCGT ATATGGGGAG ATGGTCGACA TCGCCTTTGG CAGTTGCCAT 2160

AGTTTTATCT TCTTGCACAG TAGCTGCAGT GGCGAATTTG CTCTTGACCT TCCGCACCCC 2220

TGACCGGCGA GCCTTGCACT GGACAGACAA CACACTCAGG CGCACATTGC CGCCACCCGC 2280

GATCCCCGGC GGTGCCGCCC GATCAGCATG CGAGCAAGAG CGACGATGGC CTTGGAGTCG 2340

TCGTCGGCCG GCTTGGCGGG CGACGGTGGC AACGGCACGA CGGCCTTCTC CTCGGCGATG 2400

TCCTTGGCGG CCTCCACCTC CGCCGCCGTC ATATCTATGC TATGCTACCT GCTGCTGCTG 2460

CTGCTGAATT CCCTCACTCG ATCAGCA 2487

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1865 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATGGCGGCGG CGGAGGTGGA GGCCGCCAAG GACATCGCCG AGGAGAAGGC CGTCGTGCCG 60

TTGCCACCGT CGCCCGCCAA GCCGGCCGAC GACGACTCCA AGGCCATCGT CGCTCTTGCT 120

CGCATGCTGA TCGGGCGGCA CCGCCGGGGA TCGCGGGTGG CGGCAATGTG CGCCTGAGTG 180

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CGCGGATCCC GGGTTCCAAG GGCGCCAGCG G 31

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AACTGCAGAA GGATCCCATG GTGTGCCTCG TGTCGCCC 38

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GGATGCTTAA ATGTGTACC 19

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2565 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ATGGTGTGCC TCGTGTCGCC CTCTTCCTCG CCGACTCCGC TTCCGCCGCC GCGGCGCTCT 60

CGCTCGCATG CTGATCGGGC GGCACCGCCG GGGATCGCGG GTGGCGGCAA TGTGCGCCTG 120

AGTGTGTTGT CTGTCCAGTG CAAGGCTCGC CGGTCAGGGG TGCGGAAGGT CAAGAGCAAA 180

TTCGCCACTG CAGCTACTGT GCAAGAAGAT AAAACTATGG CAACTGCCAA AGGCGATGTC 240

GACCATCTCC CCATATACGA CCTGGACCCC AAGCTGGAGA TATTCAAGGA CCATTTCAGG 300

CCTGGGAAAT ACAGAGTAGC CCTGGACTCT GATGCTCTGG TCTTCGGTGG ACATGGAAGA 2160

GTTGGCCACG ACGTGGATCA CTTCACGTCG CCTGAAGGGG TGCCAGGGGT GCCCGAAACG 2220

AACTTCAACA ACCGGCCGAA CTCGTTCAAA GTCCTTTCTC CGCCCCGCAC CTGTGTGGCT 2280

TATTACCGTG TAGACGAAGC AGGGGCTGGA CGACGTCTTC ACGCGAAACG AGAGACAGGA 2340

AAGACGTCTC CAGCAGAGAG CATCGACGTC AAAGCTTCCA GAGCTAGTAG CAAAGAAGAC 2400

AAGGAGGCAA CGGCTGGTGG CAAGAAGGGA TGGAAGTTTG CGCGGCAGCC ATCCGATCAA 2460

GATACCAAAT GAAGCCAGGA GTCCTTGGTG AGGACTGGAC TGGCTGCCGG CGCCCTGTTA 2520

GTAGTCCTGC TCTACTGGAC TAGCCGCCGC TGGCGCCCTT GGAAC 2565

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1809 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ATGGTGTGCC TCGTGTCGCC CTCTTCCTCG CCGACTCCGC TTCCGCCGCC GCGGCGCTCT 60

CGCTCGCATG CTGATCGGGC GGCACCGCCG GGGATCGCGG GTGGCGGCAA TGTGCGCCTG 120

AGTGTGTTGT CTGTCCAGTG CAAGGCTCGC CGGTCAGGGG TGCGGAAGGT CAAGAGCAAA 180

TTCGCCACTG CAGCTACTGT GCAAGAAGAT AAAACTATGG CAACTGCCAA AGGCGATGTC 240

GACCATCTCC CCATATACGA CCTGGACCCC AAGCTGGAGA TATTCAAGGA CCATTTCAGG 300

TACCGGATGA AAAGATTCCT AGAGCAGAAA GGATCAATTG AAGAAAATGA GGGAAGTCTT 360

GAATCTTTTT CTAAAGGCTA TTTGAAATTT GGGATTAATA CAAATGAGGA TGGAACTGTA 420

TATCGTGAAT GGGCACCTGC TGCGCAGGAG GCAGAGCTTA TTGGTGACTT CAATGACTGG 480

AATGGTGCAA ACCATAAGAT GGAGAAGGAT AAATTTGGTG TTTGGTCGAT CAAAATTGAC 540

CATGTCAAAG GGAAACCTGC CATCCCTCAC AATTCCAAGG TTAAATTTCG CTTTCTACAT 600

GGTGGAGTAT GGGTTGATCG TATTCCAGCA TTGATTCGTT ATGCGACTGT TGATGCCTCT 660

AAATTTGGAG CTCCCTATGA TGGTGTTCAT TGGGATCCTC CTGCTTCTGA AAGGTACACA 720

TTTAAGCATC CTCGGCCTTC AAAGCCTGCT GCTCCACGTA TCTATGAAGC CCATGTAGGT 780

ATGAGTGGTG AAAAGCCAGC AGTAAGCACA TATAGGGAAT TTGCAGACAA TGTGTTGCCA 840

CGCATACGAG CAAATAACTA CAACACAGTT CAGTTGATGG CAGTTATGGA GCATTCGTAC 900

TATGCTTCTT TCGGGTACCA TGTGACAAAT TTCTTTGCGG TTAGCAGCAG ATCAGGCACA 960

CLAIMS

What is claimed is:

1. A method of controlling the starch fine structure of starch derived from the grain of corn comprising:

(a) preparing a chimeric gene comprising a nucleic acid fragment encoding a starch branching enzyme structural gene or a fragment thereof, operably linked in either sense or antisense orientation on the upstream side to a nucleic acid fragment encoding a promoter that directs gene expression in corn endosperm tissue, and operably linked on the downstream side to a nucleic acid fragment encoding a suitable regulatory sequence for transcriptional termination,

(b) transforming corn with the chimeric gene of step (a),

wherein expression of said chimeric gene results in alteration of the fine structure of starch derived from the grain of said transformed corn compared to the fine structure of starch derived from corn not possessing said chimeric gene.

2. The method of Claim 1 wherein said alteration of starch fine structure comprises alteration of the branch chain distribution of the amylopectin molecular component of said starch.

3. The method of Claim 1 wherein said alteration of starch fine structure comprises alteration of the ratio of the amylose molecular component to the amylopectin molecular component of said starch.

4. The method of Claim 1 wherein said alteration of starch fine structure comprises alteration of the degree of polymerization of the amylose molecular component of said starch.

5. The method of Claim 1 wherein said alteration of starch fine structure comprises alteration of the branch chain distribution of the amylopectin molecular component and alteration of the ratio of the amylose molecular component to the amylopectin molecular component of said starch.

6. The method of Claim 1 wherein said alteration of starch fine structure comprises alteration of the branch chain distribution of the amylopectin molecular component and alteration of the degree of polymerization of the amylose molecular component of said starch.

7. The method of Claim 1 wherein said alteration of starch fine structure comprises alteration of the ratio of the amylose molecular component to the amylopectin molecular component and alteration of the degree of polymerization of the amylose molecular component of said starch.

8. The method of Claim 1 wherein said alteration of starch fine structure comprises alteration of the branch chain distribution of the amylopectin molecular

B4+ chains compared to the branch chain distribution of the amylopectin molecular component of starch isolated from the grain of untransformed corn.

18. Starch isolated from the grain of a corn variety prepared by the method of Claim 1 or any progeny thereof.

19. A method of preparing a thickened foodstuff comprising combining a foodstuff, water, and an effective amount of a starch of Claim 18 and cooking the resulting composition as necessary to produce said thickened foodstuff.

20. A corn variety transformed with a chimeric gene comprising a nucleic acid fragment encoding a starch branching enzyme structural gene or fragment thereof, operably linked in either sense or antisense orientation on the upstream side to a nucleic acid fragment encoding a promoter that directs gene expression in corn endosperm tissue, and operably linked on the downstream side to a nucleic acid fragment encoding a suitable regulatory sequence for transcriptional termination, or any progeny thereof.

21. A method of controlling the branch chain distribution of the amylopectin molecular component of starch in corn comprising:

(a) preparing a chimeric gene comprising a nucleic acid fragment encoding a starch branching enzyme structural gene or a fragment thereof, operably linked in either sense or antisense orientation on the upstream side to a nucleic acid fragment encoding a promoter that directs gene expression in corn endosperm tissue, and operably linked on the downstream side to a nucleic acid fragment encoding a suitable regulatory sequence for transcriptional termination,

(b) transforming corn with the chimeric gene of step (a),

wherein expression of said chimeric gene results in alteration of the branch chain distribution of the amylopectin molecular component of starch derived from the grain of said transformed corn compared to the branch chain distribution of the amylopectin molecular component of starch derived from corn not possessing said chimeric gene.

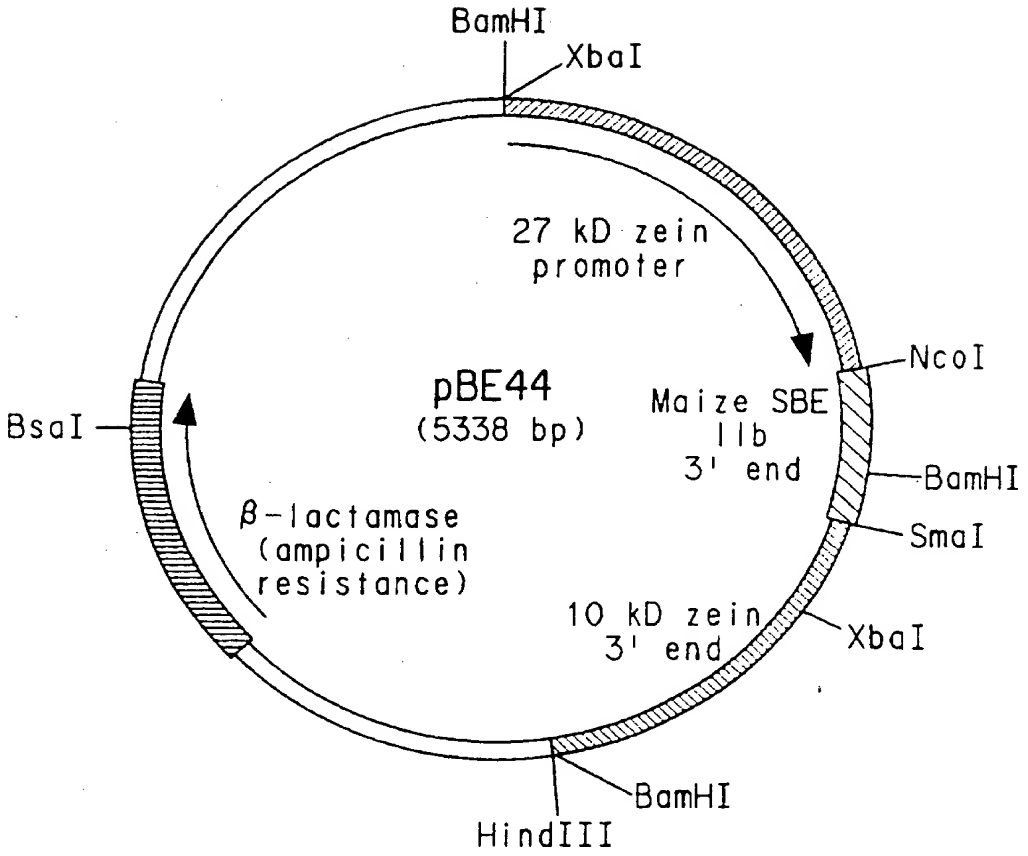

BamHI
XbaI
27 kD zein
promoter
NcoI
pBE44
(5338 bp)
Maize SBE
IIb
3' end
BamHI
SmaI
BsaI
β-lactamase
(ampicillin
resistance)
10 kD zein
3' end
XbaI
BamHI
HindIII

FIG.2

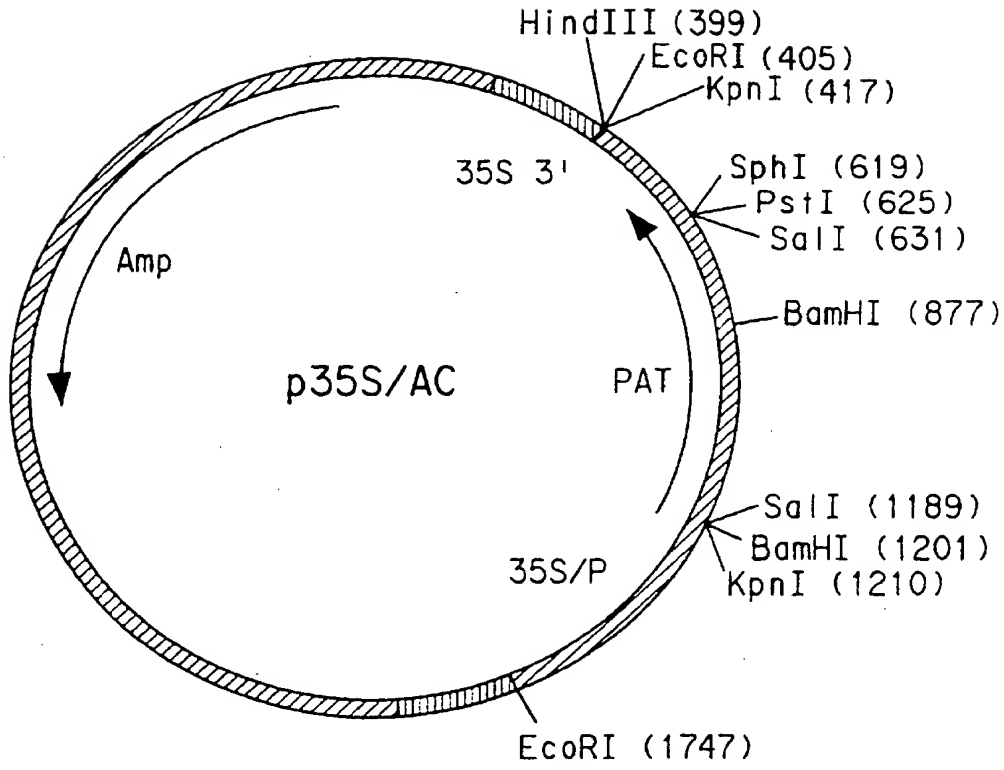


FIG.4

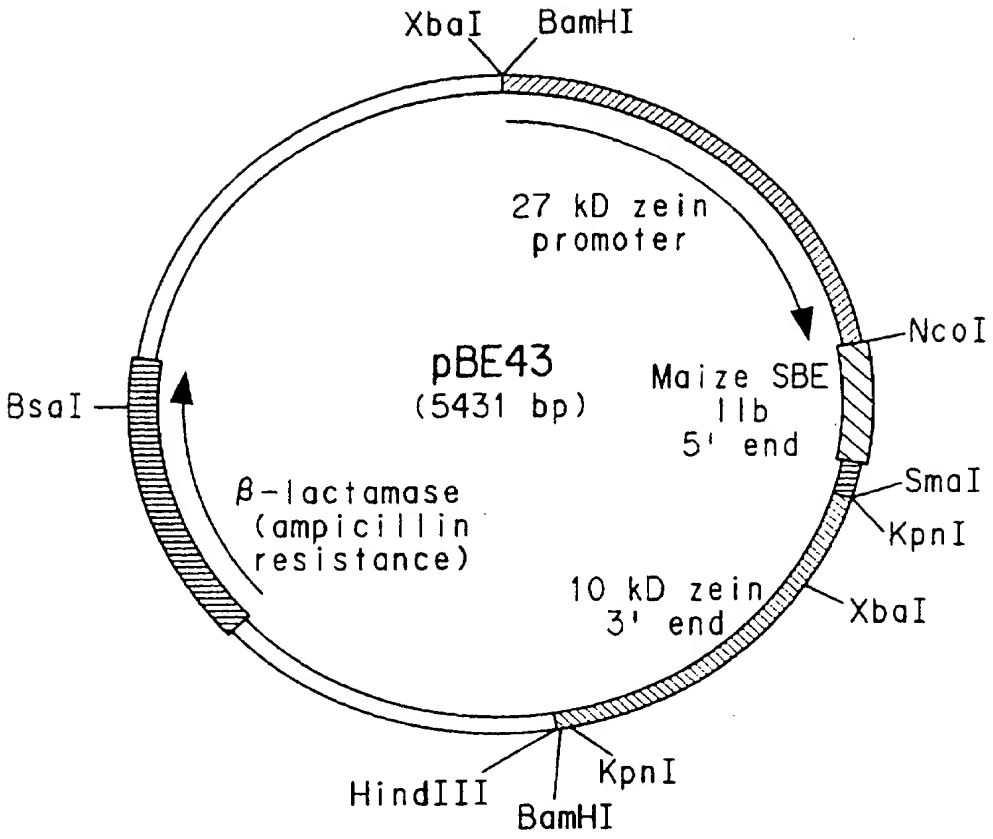

XbaI
BamHI
27 kD zein
promoter
NcoI
pBE43
(5431 bp)
Maize SBE
IIb
5' end
BsaI
SmaI
KpnI
β-lactamase
(ampicillin
resistance)
10 kD zein
3' end
XbaI
KpnI
HindIII
BamHI

FIG.6

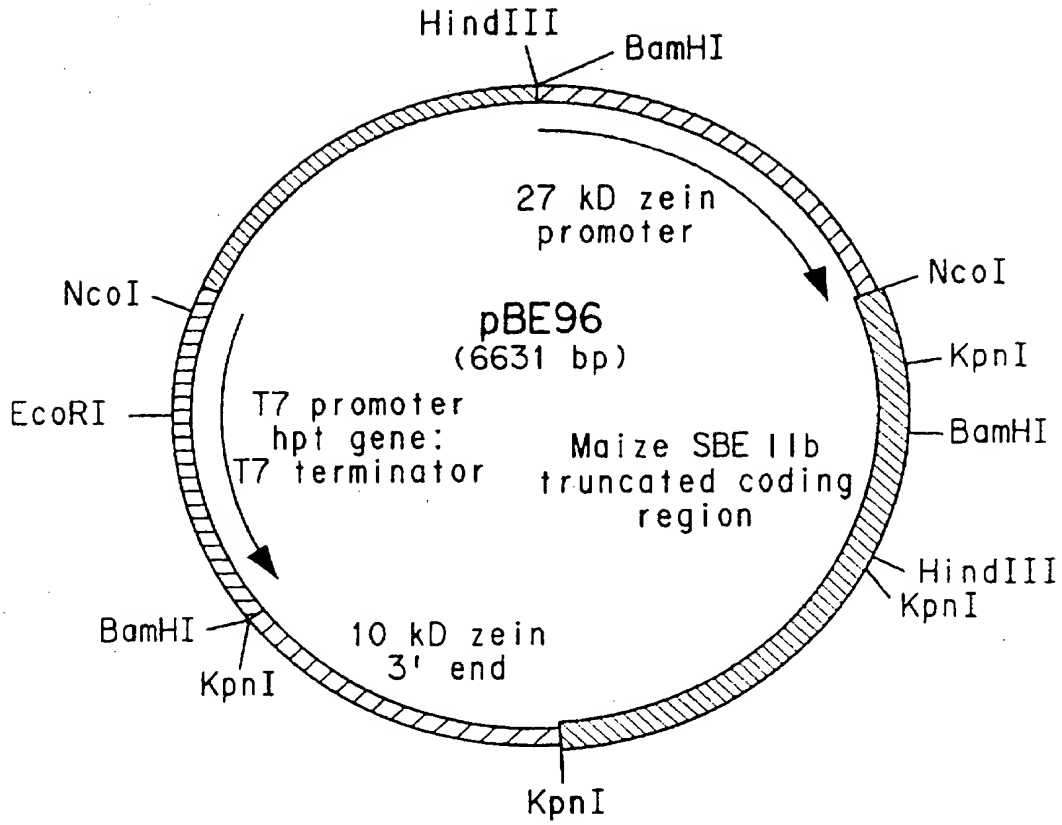


FIG.8

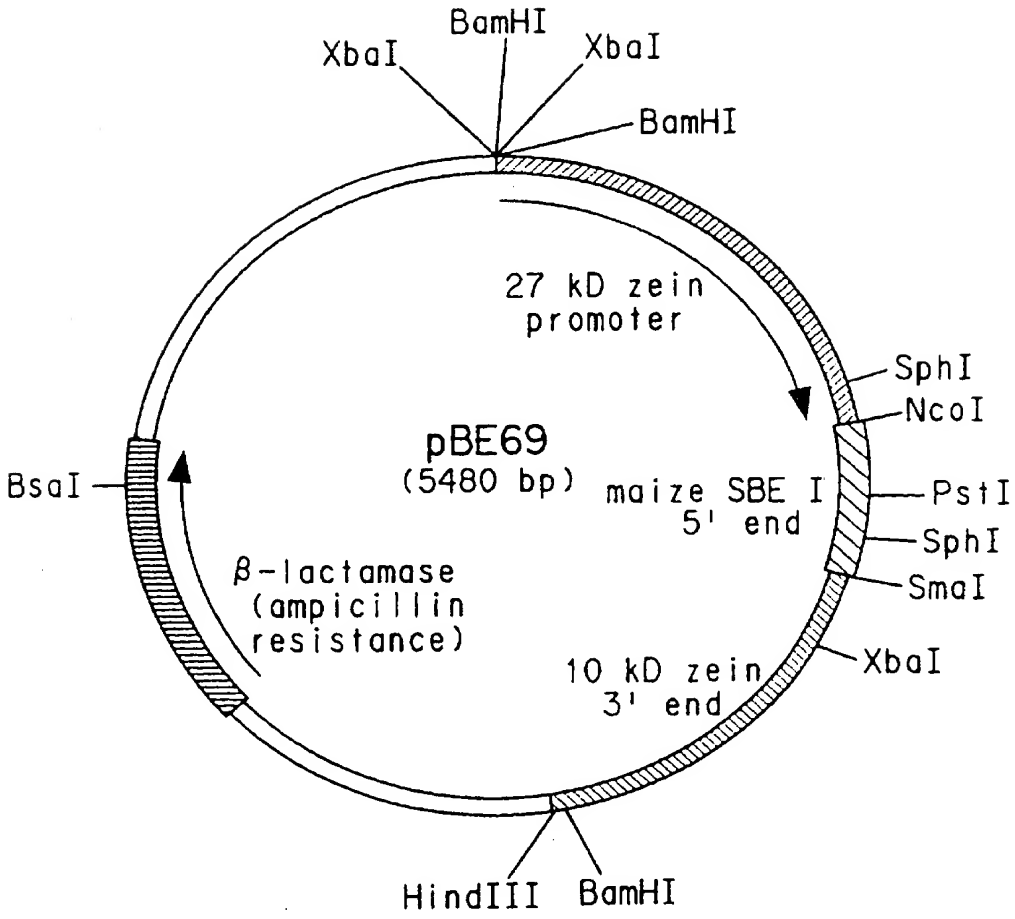

BamHI
XbaI
XbaI
BamHI
27 kD zein
promoter
SphI
NcoI
pBE69
(5480 bp)
maize SBE I
5' end
PstI
SphI
SmaI
BsaI
β-lactamase
(ampicillin
resistance)
10 kD zein
3' end
XbaI
HindIII
BamHI

FIG. 10

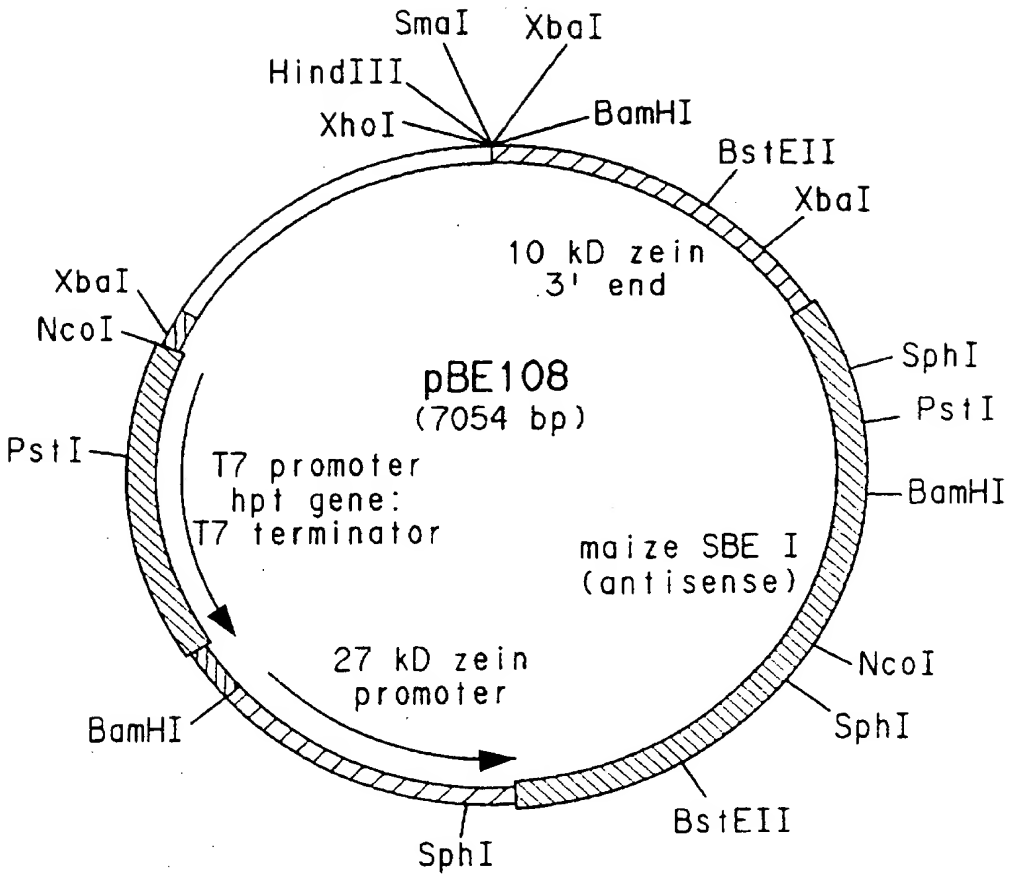


FIG. 12

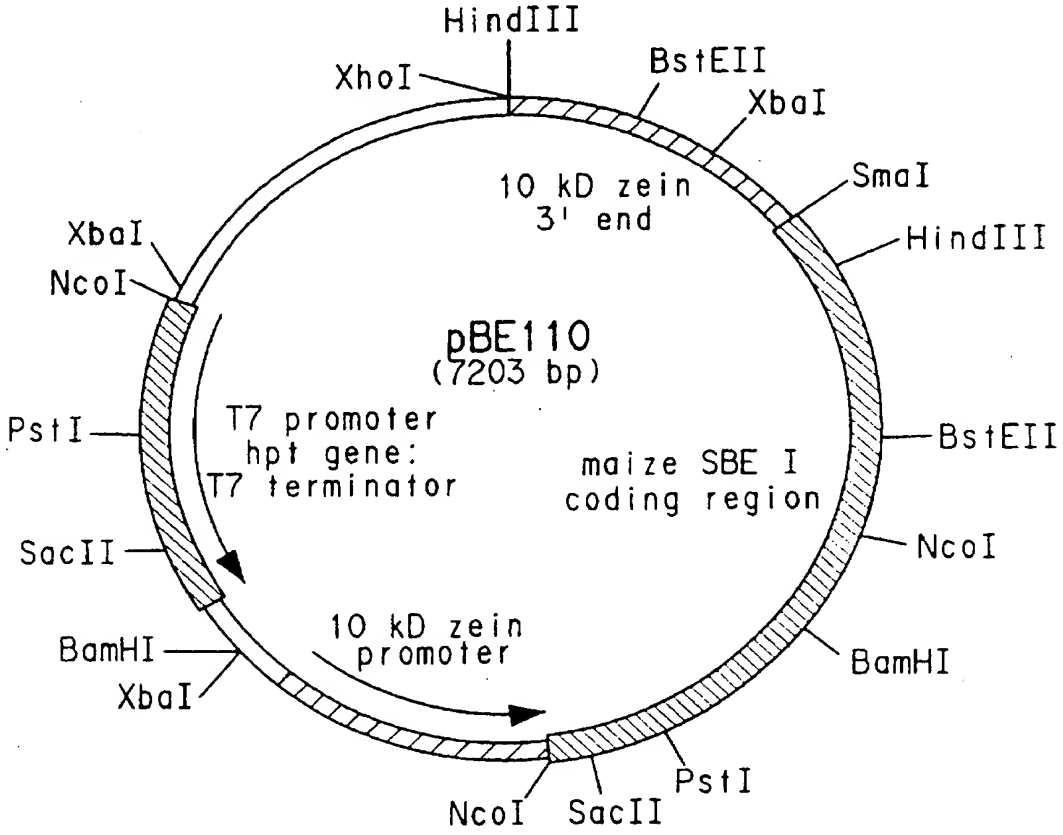
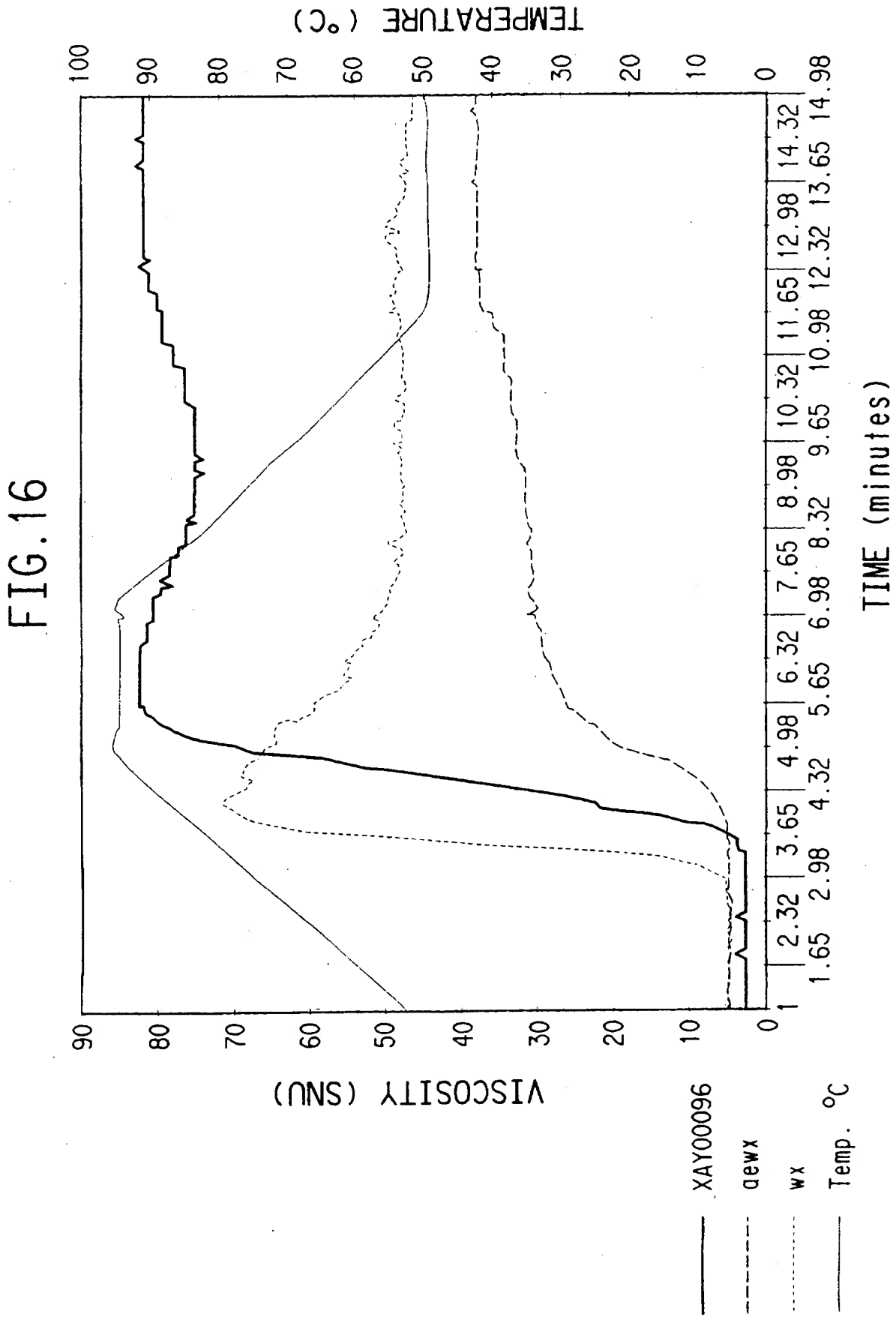


FIG. 14

FIG.16



PCT/US 96/19677

Applicant's or agent's file reference number	1066	International application No

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 22, lines 18-20

B. **IDENTIFICATION OF DEPOSIT** — Further deposits are identified on an additional sheet ☐

Name of depositary institution
AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution *(including postal code and country)*
12301 Parklawn Drive
Rockville, Maryland 20852
US

Date of deposit	Accession Number
05 December 1995 (05.12.95)	97365

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

D. **DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** *(if the indications are not for all designated States)*

E. **SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Form PCT/RO/134 (July 1992)

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/54, 15/82, 9/10, C08B 30/00, A01H 5/00, A23L 1/0522 — A3

(11) International Publication Number: WO 97/22703

(43) International Publication Date: 26 June 1997 (26.06.97)

(21) International Application Number: PCT/US96/19678

(22) International Filing Date: 12 December 1996 (12.12.96)

(30) Priority Data:
60/009,113 20 December 1995 (20.12.95) US

(71) Applicant *(for all designated States except US)*: E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: HUBBARD, Natalie, Louise [US/US]; 2204 Mill Lane, Wilmington, DE 19810-4028 (US). KLEIN, Theodore, Mitchell [US/US]; 2229 Rosewood Drive, Wilmington, DE 19810-2815 (US). BROGLIE, Karen, E. [US/US]; 520 Port Royal Court, Landenberg, PA 19350-1333 (US).

(74) Agent: MORRISSEY, Bruce, W.; E.I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).

(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, HU, IL, IS, JP, KG, KP, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, TR, TT, UA, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

With an indication in relation to a deposited microorganism furnished under Rule 13bis separately from the description.

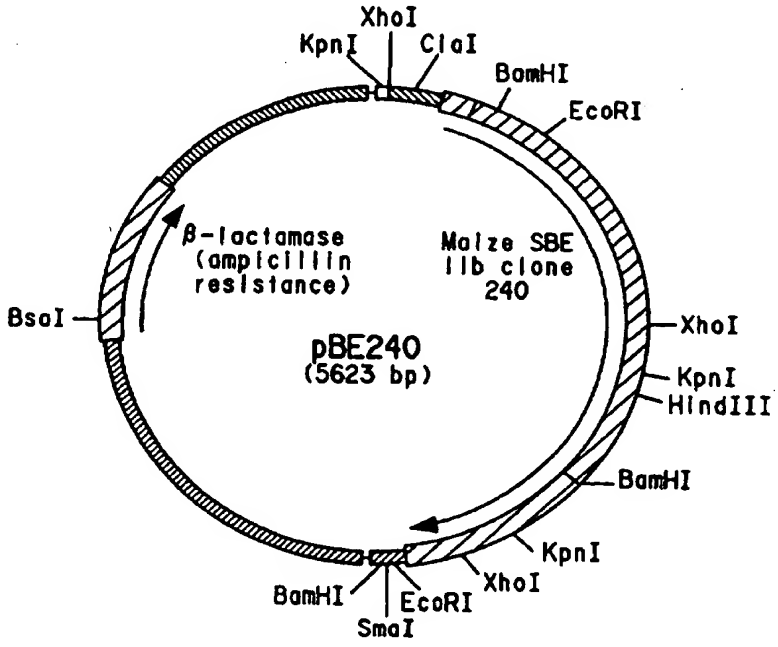
Date of receipt by the International Bureau:
25 February 1997 (25.02.97)

(88) Date of publication of the international search report:
24 July 1997 (24.07.97)

(54) Title: NOVEL STARCHES VIA MODIFICATION OF EXPRESSION OF STARCH BIOSYNTHETIC ENZYME GENES

(57) Abstract

The instant invention discloses utilization of a cDNA clone to construct sense and antisense genes for inhibition of starch branching enzyme enzymatic activity in corn. More specifically, this invention concerns a method of controlling the starch fine structure of starch derived from the grain of corn comprising: (1) preparing a chimeric gene comprising a nucleic acid fragment encoding a starch branching enzyme structural gene or a fragment thereof, operably linked in either sense or antisense orientation on the upstream side to a nucleic acid fragment encoding a promoter that directs gene expression in corn endosperm tissue, and operably linked on the downstream side to a nucleic acid fragment encoding a suitable regulatory sequence for transcriptional termination, and (2) transforming corn with said chimeric gene, wherein expression of said chimeric gene results in alteration of the fine structure of starch derived from the grain of said transformed corn compared to the fine structure of starch derived from corn not possessing said chimeric gene.



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/19678

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/54 C12N15/82 C12N9/10 C08B30/00 A01H5/00
A23L1/0522

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C08B A01H A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 11520 A (ZENECA LTD ;KEELING PETER LEWIS (GB)) 26 May 1994 see the whole document	1,9, 12-14, 18-20

X	WO 94 09144 A (ZENECA LTD) 28 April 1994 cited in the application	1,9,12, 13,18-20
Y	* see the whole document, esp. p.10,l.1-10, p.34,l.2-17, p.53,l.13-35 *	2-8,10, 11, 14-17,21

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
28 May 1997	10.06.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Kania, T

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PLANT PHYSIOLOGY, vol. 104, 1994, pages 1449-1453, XP002031588 GUAN H. ET AL.: "Expression of branching enzyme I of maize endosperm in Escherichia coli" * see the whole document, esp. p.1452, last chapter *	1-21
A	P.N.A.S., U.S.A., vol. 92, no. 4, 14 February 1995, pages 964-967, XP002031589 GUAN H. ET AL.: "Maize branching enzyme catalyzes synthesis of glycogen-like polysaccharide in glgB-deficient Escherichia coli" see the whole document	1-21